



Original Articles

Expression of Prostate-Specific Membrane Antigen in Normal, Benign, and Malignant Prostate Tissues

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Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein recognized by the murine monoclonal antibody (MAb) 7E11-C5.3 both in its native (CYT-351) and immunconjugate form (CYT-356). Previous studies have shown that tissue expression of PSMA is highly restricted to prostate tissues. In this study, a definitive immunohistochemistry evaluation was performed to assess PSMA expression in prostate tissues. A stain index was established by multiplying the percentage of stained cells by the intensity of the stained cells to provide a quantitative measurement of PSMA expression in the various tissue types. The cellular location of PSMA, its correlation with clinical status, and its comparison with the expression of prostate-specific antigen (PSA) were evaluated. Prostate-specific membrane antigen was found to be highly expressed in most of the normal intraepithelial neoplasia, and the primary and metastatic prostate tumor specimens evaluated. In contrast to PSA, PSMA expression was often heterogeneous with variable staining patterns, ranging from a low-level diffuse cytoplasmic staining in normal prostate epithelium to very intense cytoplasmic and focal membrane staining in high-grade primary carcinomas and metastatic tissues. The predominant cytoplasmic staining was expected because the antigenic epitope of the PSMA transmembrane glycoprotein recognized by MAb 7E11-C5.3 is located in the cytoplasmic domain. Benign prostate tumors, ie, hypertrophy, showed the lowest expression of PSMA with a stain index of 52, compared with stain indexes of 146 and 258 for normal prostate and bone metastatic tissues, respectively. The reason for the apparent down-regulation of PSMA in benign prostate tissue is unknown but may be related to a splicing variant or post-translational modification of PSMA. Expression of PSMA was observed to increase with increasing pathologic grade, but not with clinical stage. Although PSMA was overexpressed in poorly differentiated and metastatic prostate tumors, expression in the primary tumor did not correlate with nodal status, extracapsular penetration, or seminal vesicle invasion. These results suggest that PSMA is not a

useful biomarker of disease progression; however, high expression does appear to be associated with the more aggressive prostate carcinoma phenotype. The restricted specificity, differential prostate tissue expression, and overexpression of PSMA in metastatic tissues support the continued study of this unique prostate tumor-associated biomarker for developing new strategies for diagnosis and therapy of prostate cancer. (*Urol Oncol* 1995;1:18-28)

Prostate cancer is the most common (noncutaneous) cancer diagnosed in the American male and is steadily increasing, not only as a result of an increasing population of older men, but also because of greater awareness of the disease and earlier diagnosis using tumor markers such as prostate-specific antigen (PSA). It is projected that 200,000 men were diagnosed with prostate cancer in 1994,¹ representing a 34% increase in the number of prostate cancer cases (165,000) diagnosed in 1993. If the 1994 estimate is accurate, prostate cancer will become the most commonly diagnosed cancer, exceeding breast cancer (183,000) by 27,000 cases. More than 38,000 men are expected to die of prostate cancer in 1994, making deaths from prostate cancer second only to lung cancer deaths. Patients diagnosed with localized disease have far better survival rates than patients diagnosed with metastatic disease. Early detection of localized prostate cancer and improved treatment of metastatic disease are important strategies to reduce prostate cancer deaths.

Although serum PSA measurements have had a major impact on the diagnosis and management of prostate cancer,^{2,3} PSA is far from being the ideal cancer marker. Twenty-five percent of patients with benign prostatic hypertrophy (BPH) present with elevated levels of PSA, approximately 30% of prostate cancer patients present with normal PSA values, and PSA expression is unable to differentiate biologically active from inactive cancers. These statistics suggest that other clinical markers are needed to improve early diagnosis, to identify aggressive tumors, and to develop new therapeutic strategies. A new prostate marker, prostate-specific membrane antigen (PSMA), may meet one or more of these objectives. Prostate-specific membrane antigen appears to be a transmembrane glycoprotein with a major Mr 100,000 component⁴⁻⁶ recognized by monoclonal antibody (MAb) 7E11-C5.3.⁷ Recently, the cDNA encoding PSMA was cloned, and the deduced amino acid sequence revealed a novel polypeptide

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structure.⁴ Clinical trials using MAb 7E11-C5.3 conjugated to either ¹¹¹In or ⁹⁰Y are in progress for diagnostic imaging and therapy, respectively.⁸⁻¹¹ Previous reports^{5,7-9} have shown that PSMA expression is highly restricted to prostate tissues and that the expression in normal prostate tissues appeared to be less than that in malignant prostate tissues. These studies, performed primarily to determine the specificity of PSMA expression, evaluated a small number of tissue specimens, and no relation of PSMA expression to clinical status was presented. In this report, we present a definitive description of the differential expression of PSMA in normal prostate, BPH, prostate intraepithelial neoplasia (PIN), and primary and metastatic prostate carcinoma (CaP) tissues, and the relation of PSMA expression to tumor grade and extraprostatic disease.

Materials and Methods

Tissues

Formalin-fixed, paraffin-embedded blocks of transurethral resected specimens of BPH, prostatectomy specimens of prostate carcinoma, and lymph node and bone metastatic tissues were obtained from the Virginia Prostate Center Tissue Bank. Normal prostate tissue was obtained from males aged 16 to 45 years with no evidence of prostate disease. These tissues were obtained by autopsy, usually within 12 hours after death, from the Cooperative Human Tissue Network, University of Alabama at Birmingham; and from the Norfolk Medical Examiner's Office. All tissue specimens for paraffin embedding were fixed in the same neutral buffered 10% formalin. Samples of normal, BPH, and prostate carcinoma tissues obtained directly from surgery or autopsy also were embedded in OCT compound in cryomolds and snap-frozen in isopentane over liquid nitrogen.

Monoclonal Antibodies

Affinity-purified MAb 7E11-C5.3 (referred to as native antibody or CYT-351) and an affinity-purified conjugated form of MAb 7E11-C5.3 (designated CYT-356¹²) were provided by CYTOGEN Corp. (Princeton, NJ). Monoclonal antibody EVMS-PSA-5 was produced in mice against purified PSA from pooled normal seminal plasma and affinity purified from ascites, following our published protocols.¹³⁻¹⁵

Immunoperoxidase Staining

The expression of PSMA and PSA in tissues was detected by the avidin-biotin peroxidase assay using the ABC Elite Vectastain kit (Vector Laboratories, Burlingame, CA), as described previously.¹³⁻¹⁵ Briefly, 4- μ m paraffin sections were cut, deparaffinized, and rehydrated through xylene and a graded series of alcohols. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 30 minutes. Frozen sections were cut at 6 μ m, briefly dipped in cold acetone, and stored at -20°C until used, or air dried for 30 minutes before proceeding with the staining reaction. Endogenous peroxidase activity was blocked with 3.0% H₂O₂ in water for 5 minutes.

From this point on, both frozen and paraffin-embedded sections were treated in the same manner. Nonspecific binding was blocked by incubation with 10% normal horse serum for 10 minutes, followed by a 30-minute incubation with the primary antibody, either MAb 7E11-C5.3 or PSA-5, followed by a 10-minute incubation with the biotinylated secondary antibody, and then the ABC complex. The optimal concentrations for the antibodies (20 μ g/mL for MAb 7E11-C5.3 and 2 μ g/mL for PSA-5) were predetermined by titrating the MAbs on normal, BPH, and prostate carcinoma tissues. These concentrations from the same antibody lot were used for evaluating all the specimens in this study. After development with the chromogen substrate 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO), the sections were counterstained with Mayer's hematoxylin and mounted in aqua mount (Learner Laboratories, Pittsburgh, PA). Antigen expression was scored by calculating the percentage of cells positive in a number of 20-mm objective views sufficient to cover all areas of the tissue section. Staining intensity was also recorded using a scale of 1 (low), 2 (moderate), and 3 (high). The stained tissue sections were scored independently by two investigators, with the two scores having a difference of less than 10%. A stain index was calculated by multiplying the mean percentage PSMA-positive cells by the mean staining intensity.

Results

Expression of PSMA and PSA in Normal, Benign, and Malignant Prostate Tissues

The binding of MAb 7E11-C5.3 was compared with the binding of a MAb to PSA using the immunoperoxidase assay on frozen and formalin-fixed, paraffin-embedded tissue sections of prostate specimens consisting of normal prostate, BPH, and malignant primary and metastatic prostate carcinomas. By optimizing the staining conditions, both frozen (data not shown) and paraffinized tissue specimens from the same patient gave identical staining patterns for MAb PSA-5 and the native and immunoconjugate forms of MAb 7E11-C5.3. The use of paraffin-embedded tissues enabled us to conduct a large retrospective study to evaluate MAb 7E11-C5.3 reactivity on normal, benign, PIN, and malignant prostate tissues. The epithelial cells of all four prostate tissue types were found to express both the PSMA and PSA antigens (Table 1; Figures 1-4). As expected, nearly all the prostate specimens expressed PSA, with the exception of the bone marrow metastatic specimens, of which only 57% stained positive for PSA. The mean percentage of epithelial cells expressing PSA ranged from 80-98% for all prostate tissue types, with the exception of the bone metastatic specimens (48%). Ninety-one to 100% of the prostate tissues expressed PSMA, with the exception of the BPH specimens, in which PSMA expression was positive in only 22 of 27 (81%). The mean percentage of epithelial cells expressing PSMA in the PIN specimens was 59%, and in the primary prostate carcinomas was 53%. The highest expression was found in the metastatic tumors: 72% for lymph nodes and 92% for bone metastasis. Of special interest was the observation that the highest percentage

TABLE 1. COMPARISON OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN WITH PROSTATE-SPECIFIC ANTIGEN EXPRESSION IN FORMALIN-FIXED, PARAFFIN-EMBEDDED PROSTATE TISSUES

Tissue	PSMA		PSA	
	No. positive/ no. tested	Mean % positive cells	No. positive/ no. tested	Mean % positive cells
Normal	12/12 (100%)	77	12/12 (100%)	98
BPH	22/27 (81%)	29	27/27 (100%)	95
PIN	21/21 (100%)	59	21/21 (100%)	98
CaP	157/165 (95%)	53	161/165 (98%)	81
LN mets	72/79 (91%)	72	74/79 (94%)	81
Bone mets	7/7 (100%)	92	4/7 (57%)	48

PSMA = prostate-specific membrane antigen; PSA = prostate-specific antigen; BPH = benign prostate hyperplasia; PIN = prostate intraepithelial neoplasia; CaP = primary prostate carcinoma; LN = lymph node; Mets = metastasis.

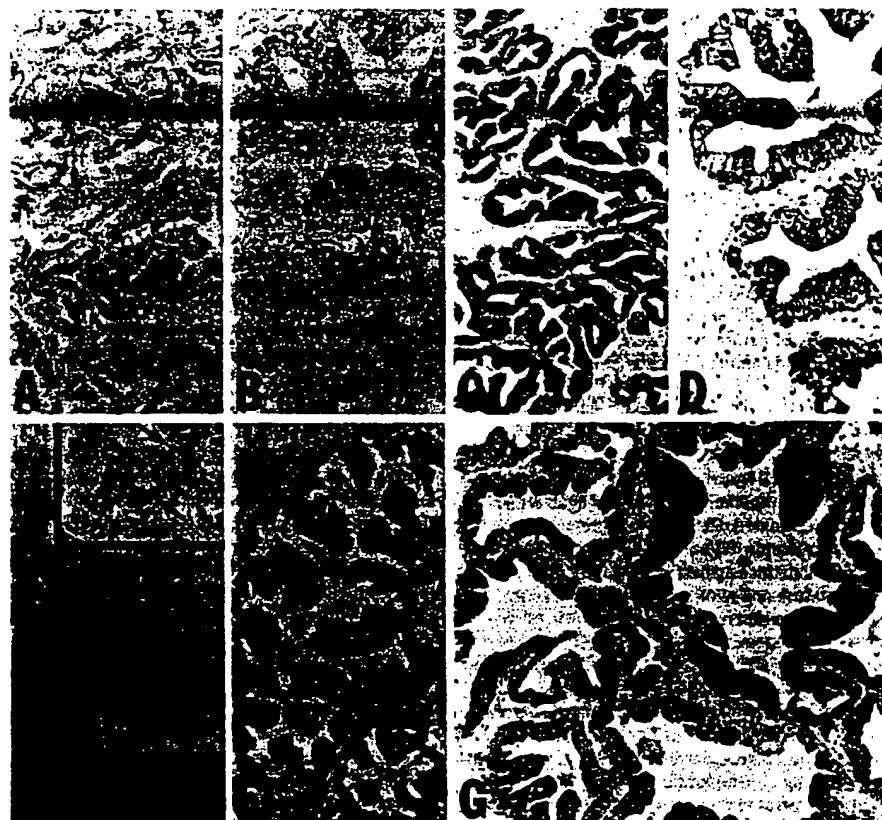


FIGURE 1. Immunoperoxidase staining of normal, benign (BPH), and PIN tissues with monoclonal antibodies to either PSMA or PSA. A-D) Normal prostate tissues. A) and B) stained for PSMA; C) and D) stained for PSA. Note diffuse cytoplasmic and low staining intensity for PSMA. E) and F) BPH tissue stained for PSMA (E) and PSA (F). Note nuclear staining for PSMA (arrows). G) PIN stained for PSMA. Original magnification: $\times 100$ (A,C); $\times 200$ (B,D,E,F,G); $\times 400$ (E inset).

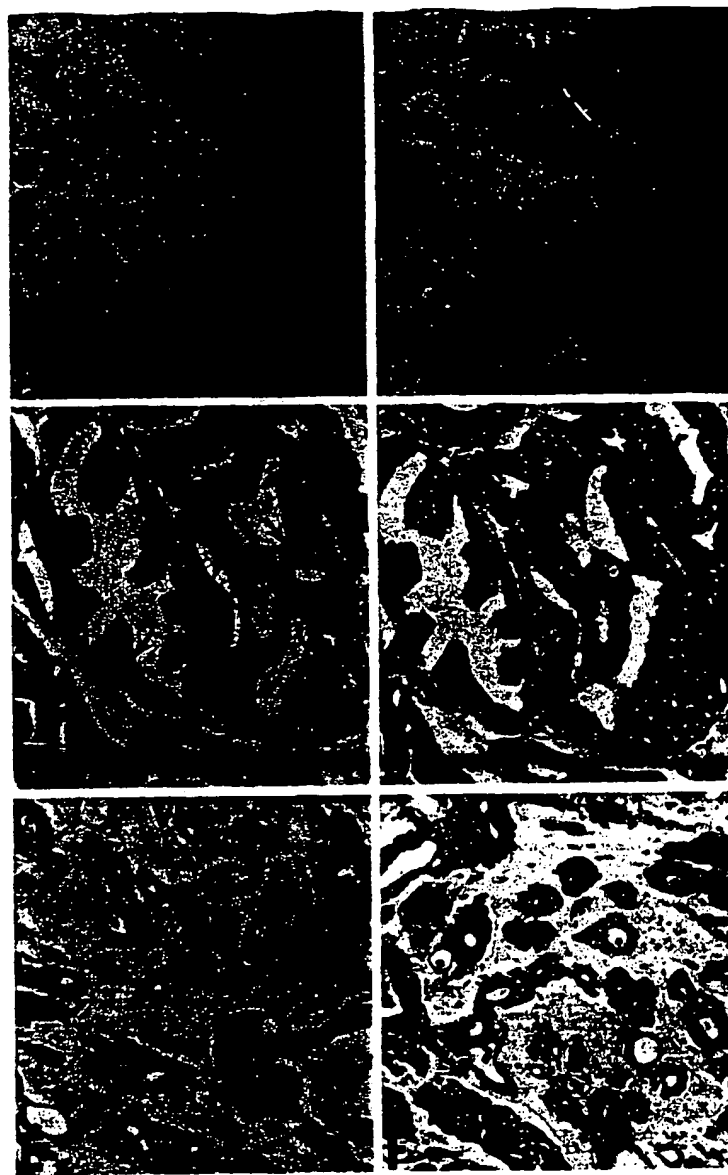


FIGURE 2. Prostate carcinoma tissues stained with monoclonal antibody to either PSMA or PSA. A) and B) Well-differentiated carcinoma; C) and D) moderately differentiated carcinoma; E) and F) poorly differentiated carcinoma. A), C), and E) stained for PSMA; B), D), and F) stained for PSA. Note the minimal and low-intensity cytoplasmic staining for PSMA in the well-differentiated carcinoma, with increase in the number of cells and staining intensity with increasing tumor grade, and increase focal and luminal membrane staining (arrows). Original magnification: $\times 200$ (A-F).

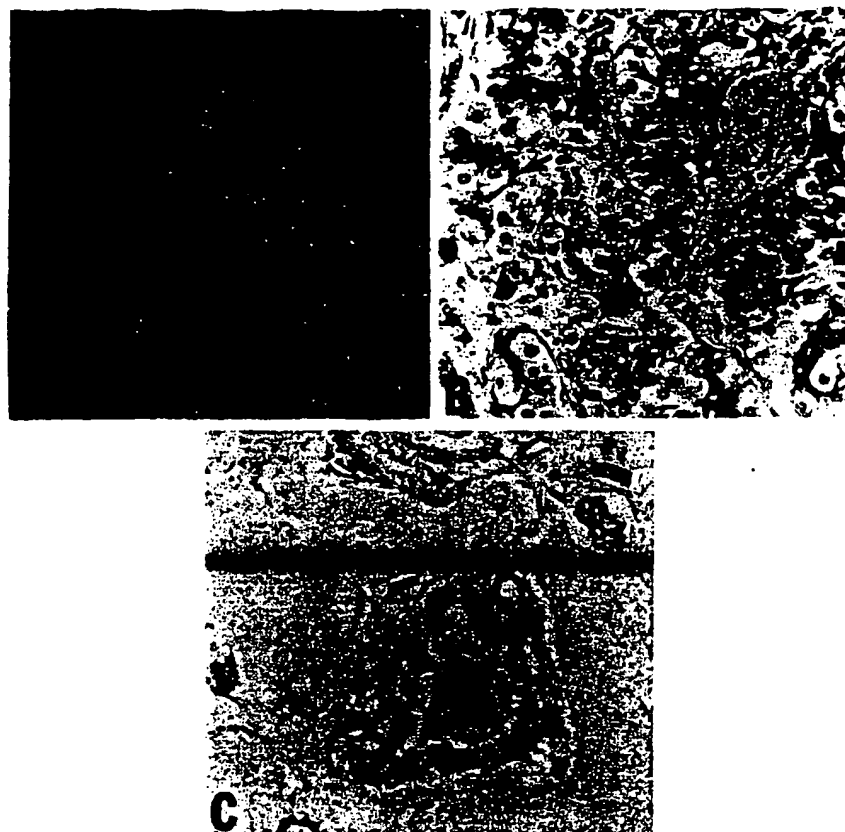


FIGURE 3. Metastatic prostate tissues stained for PSMA. A) and B) Lymph node metastatic tissue. C) Bone metastatic tissue. B) is a higher magnification showing intense focal and luminal membrane staining (arrows) associated with minimal cytoplasmic staining. Original magnification: $\times 100$ (A,C); $\times 400$ (B).

(77%) of epithelial cells expressing PSMA was in the normal prostate specimens, and the lowest percentage of positive cells (29%) occurred in the BPH specimens.

Expression and Cellular Localization of PSMA in Prostate Tissues

We evaluated PSMA expression in prostate tissues further by taking into consideration both the percentage and the staining intensity of prostate epithelial cells. A stain index was calculated by multiplying the mean percentage of cells expressing PSMA by the staining intensity (1 = low; 2 = moderate; 3 = high intensity). With this approach, BPH specimens clearly had the lowest stain index (SI = 52), ie, the lowest number of positive epithelial cells and the lowest staining intensity (Table 2, Figure 1E). Both the number of stained cells and the staining intensity increased in the PIN (Figure 1G) and the malignant prostate specimens (Figure 2), with the highest index (SI = 258) determined for the bone metastatic

specimens. Although the normal prostate tissues had a high stain index (SI = 146), the staining pattern was quite different from that of the other tissue specimens. In these specimens, PSMA expression was predominantly diffuse and cytoplasmic with low to moderate staining intensity (Table 2; Figure 1B), with an occasional duct or luminal cell showing luminal membrane staining. Of all prostate tissues examined, BPH demonstrated the greatest antigenic heterogeneity. In contrast to normal prostate, few luminal cells expressed PSMA and the expression was often focal, with some apical membrane staining and minimal cytoplasmic staining (Figure 1E). The PIN lesions had a stain index (SI = 130) similar to normal prostate but showed a more intense, diffuse cytoplasmic and luminal membrane staining (Figure 1G). The stain index (SI = 133) for PSMA expression in the 165 primary prostate carcinomas evaluated, including all histopathologic grades, was similar to the index for normal and PIN tissues (Table 2). Cellular localization of PSMA in these tissues ranged from diffuse cytoplasmic staining in the well-differentiated speci-

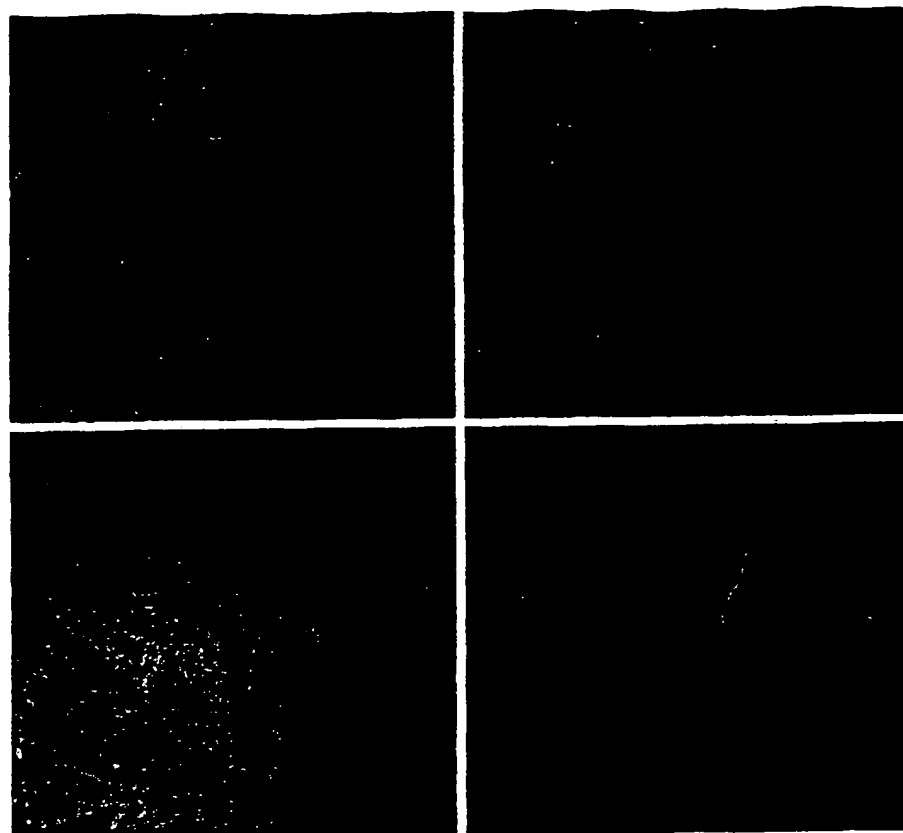


FIGURE 4. Immunoperoxidase staining of three representative prostate carcinomas to illustrate the differential PSMA expression in various tissue types in the same specimen. A) and B) are serial sections from one patient; C) and D) are sections from two different patients, respectively. A), C), and D) were stained for PSMA and B) was stained for PSA. Note minimal to no staining in the normal/benign and well-differentiated (arrows) areas, compared with the intense focal and luminal membrane staining with some cytoplasmic staining (particularly in section D) in the poorly differentiated carcinoma areas. Note that all tissue areas stained for PSA (B). Original magnification: $\times 100$ (A-D).

TABLE 2. DIFFERENTIAL EXPRESSION OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN IN FORMALIN-FIXED, PARAFFIN-EMBEDDED PROSTATE TISSUES

Tissue	No. positive/ no. tested	% positive cells ^a	Intensity ^a	Stain index ^b	PSMA localization ^c			
					DC	FC	F/M	LE
Normal	12/12 (100%)	77 \pm 32	1.9 \pm 0.33	146	++			+
BPH	22/27 (81%)	29 \pm 29	1.8 \pm 0.90	52	+		++	+
PIN	21/21 (100%)	59 \pm 21	2.2 \pm 0.37	130	+		++	
CaP	157/165 (95%)	53 \pm 32	2.5 \pm 0.66	133	++		++	+++
LN mets	60/64 (94%)	72 \pm 36	2.7 \pm 0.92	194	+	++	+++	+++
Bone mets	7/7 (100%)	92 \pm 10	2.8 \pm 0.40	258	+	++	+++	+++

DC = diffuse cytoplasmic; FC = focal areas within the cytoplasm; F/M = membrane or focal membrane; LE = edge of luminal cells; other abbreviations as in Table 1.

^aMean \pm SD.

^bStain index calculated by multiplying the mean percentage of cells staining by the staining intensity.

^c++ = low staining; +++ = moderate staining; ++++ = high staining.

TABLE 3. PROSTATE-SPECIFIC MEMBRANE ANTIGEN EXPRESSION VERSUS GRADE

Gleason sum	No. positive/ no. tested	Mean % cells positive	Mean stain intensity	Stain index
2-4 (WD)	22/26 (85%)	48	2.3	110
5-7 (MD)	92/100 (92%)	50	2.5	125
8-10 (PD)	37/39 (95%)	62	2.6	161

WD = well-differentiated; MD = moderately differentiated; PD = poorly differentiated.

mens (Figure 2A) to intense focal and luminal membrane staining in the moderate (Figure 2C) to high-grade carcinomas (Figure 2E). Perinuclear staining was sometimes observed in a few malignant epithelial cells in some of the high-grade specimens. Staining heterogeneity was evident for all tumor grades, with the highest degree observed in the low to moderate grades. The highest PSMA expression, in terms of number of cells staining and intensity of staining, was observed in the metastatic tissues (Table 2; Figure 3). As shown in Figure 3, the predominant cellular expression of PSMA in the lymph node and bone metastatic specimens was intense apical membrane staining, often with minimal cytoplasmic staining (Figure 3). Less common was the finding of occasional perinuclear staining and intensely stained focal areas

within the cytoplasm (not shown). The PSMA staining pattern observed in the metastatic tissues was similar to that for the moderate to high-grade prostate carcinoma specimens (Figure 2C and E); however, there was considerable less staining heterogeneity in the metastatic tissues.

PSMA Expression by Tumor Grade

Table 3 shows the expression of PSMA in the different pathologic grades. The Gleason scores of all 165 primary prostate carcinomas were placed into three groups representing the three general pathologic differentiation grades. Although not remarkable, a slight but positive correlation of PSMA expression with tumor grade was observed. The differential expres-

TABLE 4. COMPARISON OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN EXPRESSION IN LOW-GRADE AND HIGH-GRADE TUMOR AREAS ON THE SAME PROSTATE CARCINOMA TISSUE SPECIMEN

Tissue specimen	Low grade			High grade		
	%Cell ^a	Int ^b	Index ^c	%Cell ^a	Int ^b	Index ^c
CA153	20	1	20	95	3	285
CA1061	20	1	20	98	2.5	245
CA8750	10	1	10	70	3	210
CA5262	20	1	20	75	3	225
CA1124	70	3	210	90	3	270
CA1197	35	1	35	100	3	300
CA1789	50	1	50	50	2	100
CA8331	20	1	20	98	3	294
CA1022	5	1	5	90	2.5	225
CA2048	55	3	165	100	2	200
CA7506	70	2	140	95	3	285
CA4319	5	1	5	90	2	180
CA4475	35	2	70	90	3	270
CA3651	10	2	20	80	3	240
CA3984	20	1	20	60	3	180
CA8725	50	3	150	90	3	270
CA1471	85	2	170	100	3	300
CA6166	10	1	10	95	3	285
CA0600	40	2	80	70	3	210
CA5850	100	1	100	100	3	300
CA1977	65	3	195	80	3	240
CA1709	10	1	10	99	2.5	248

^a%Cell = percentage of tumor cells staining.^bInt = staining intensity (1 = low; 2 = moderate; 3 = strong).^cStaining index, calculated by multiplying the mean percentage of positive cells by staining intensity.

sion of PSMA in low- and high-grade carcinomas was more accurately assessed when separate indexes were calculated for the low- and high-grade areas contained in the same tumor specimen. Table 4 shows the results of PSMA expression in 22 randomly selected high-grade carcinomas containing focal areas of low-grade carcinoma. In all 22 cases, the stain index for the high-grade areas (mean SI = 244) was higher than that for the low-grade areas (mean SI = 59). Figure 4 shows the differential staining patterns in three carcinoma specimens containing both high- and low-grade tumor areas. Strong staining was observed in the poorly differentiated areas, often with minimal to no staining of the low-grade areas and the normal/benign areas. No correlation was found between PSMA expression and clinical or pathologic stage (data not shown).

PSMA Expression in Lymph Node Metastases

The high PSMA expression in metastatic lymph nodes (94% of 64 positive nodes, SI = 194; Table 2) suggested that PSMA

expression in primary carcinomas may represent a biomarker of metastatic progression. Although the percentage of tumor cells expressing PSMA often was increased in the metastatic lymph node (Table 5), the patient's primary carcinoma did not reflect this PSMA activity; therefore, PSMA staining in the primary tumor was not predictive of nodal status (Table 6). Similarly, PSMA expression did not correlate with positive margins, extracapsular penetration, or seminal vesicle invasion (data not shown). Correspondingly, PSA expression also did not correlate with these pathologic parameters (data not shown).

Discussion

Prostate-specific membrane antigen is expressed as a prominent Mr 120,000 transmembrane glycoprotein in prostate tissue extracts and seminal plasma.⁴⁻⁶ It is detected using the mouse Mab 7E11-CS3, produced against a membrane extract of LNCaP cells.^{7,16,17} Immunostaining of normal and malignant tissues demonstrated that PSMA expression is highly restricted to prostate tissues^{7,12} (also Wright GL Jr, Haley C.

TABLE 5. EXPRESSION OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND PROSTATE-SPECIFIC ANTIGEN IN THE PATIENT'S PRIMARY PROSTATE CARCINOMA AND LYMPH NODE METASTASIS

Patient	Primary carcinoma			Metastatic lymph nodes	
	Gleason ^a sum	Percent cells		Percent cells	
		PSMA	PSA	PSMA	PSA
CA2577	4	0	90	10	25
CA1290	4	5	40	20	70
CA5624	5	70	100	5	5
CA4306	5	10	95	75	100
CA2149	6	5	90	0	95
CA2071	7	15	100	45	95
CA9970	7	40	95	95	95
CA1842	7	40	10	75	25
CA5972	7	85	98	98	93
CA6918	7	20	100	100	90
CA4495	7	95	100	100	100
CA5371	7	25	10	5	30
CA8170	7	45	100	90	50
CA1197	7	00	95	100	100
CA1064	7	60	85	65	100
CA6136	8	65	100	40	100
CA1007	8	90	100	80	95
CA1435	8	70	100	100	90
CA1640	8	10	40	95	50
CA1602	8	55	90	95	80
CA4475	8	75	100	100	100
CA1360	8	5	100	5	75
CA1551	8	85	90	75	95
CA5750	8	80	100	100	90
CA8292	8	75	90	25	25
CA3411	9	30	35	25	80
CA3984	9	70	100	90	100

PSMA = prostate-specific membrane antigen; PSA = prostate-specific antigen.

^aGleason sum: 1-4 = well-differentiated; 5-7 = moderately differentiated; 8-10 = poorly differentiated.

TABLE 6. PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND PROSTATE-SPECIFIC ANTIGEN EXPRESSION IN PRIMARY CARCINOMA VERSUS NODE STATUS

Node status	PSMA			PSA		
	No. tested	No. positive	Mean % positive cells*	No. tested	No. positive	Mean % positive cells*
Positive	21	21	54 \pm 32.1	21	21	86 \pm 26.2
Negative	78	75	49 \pm 32.0	78	77	76 \pm 27.0

Abbreviations as in Table 3.

*Mean \pm SD.

Beckett ML, unpublished results). Early immunohistochemistry studies showed that Mab 7E11-C5.3 bound the type-2 muscle fibers of normal skeletal muscle; however, a ^{111}In -labeled immunoconjugate (CYT-356) of Mab 7E11-C5.3 failed to localize to skeletal muscle.¹² Recent studies in our laboratory (Troyer JK, Feng Q, Beckett ML, Wright GL Jr, unpublished results) and at Sloan-Kettering¹⁸ have shown that neither the PSMA glycoprotein nor the PSMA mRNA could be detected in tissue extracts of normal skeletal muscle, suggesting that the observed immunostaining in skeletal muscle is entirely non-specific. Further studies from these laboratories have shown mRNA¹⁸ and PSMA (Troyer et al, unpublished results) in extracts of normal brain, salivary gland, and small intestine by blotting procedures, but not by immunohistochemistry of frozen or formalin-fixed tissue sections.^{7,12} (also Wright et al, unpublished results). These results suggest either that PSMA expression is below the detection limits of the immunohistochemistry assay or that post-translational modifications mask the PSMA epitope in these tissues. Previous immunohistochemistry studies focused on evaluating the specificity of Mab 7E11-C5.3 tissue reactivity. The present study provides a definitive descriptive immunohistochemistry examination of PSMA expression in normal, benign, and malignant prostate tissues.

Immunoreactivity for PSMA was detected in all types of prostate epithelium, confirming organ specificity rather than prostate carcinoma specificity of this biomarker. Expression of PSMA and PSA in all tissue specimens, with the exception of BPH and bone metastases, paralleled each other. Expression of PSMA in the majority of BPH specimens appeared to be both very heterogeneous and down-regulated. These immunostaining results correlate with the recent observation that PSMA mRNA levels are low to absent in BPH, even though they are high in both normal and malignant prostate tissues.¹⁸ The reason for this phenomenon has yet to be determined. Recent evidence localizing the antigenic epitope recognized by Mab 7E11-C5.3 in the cytoplasmic domain of the PSMA glycoprotein (see Troyer et al, this issue)¹⁹ may indicate that a splicing variant involving the N-terminal amino acid sequence could be responsible for the low PSMA expression in BPH tissues. New antibodies to different PSMA epitopes may assist in addressing this question. Further studies will be required to evaluate this or alternative hypotheses to explain the low PSMA expression in BPH tissues.

The pattern and localization of immunostaining were variable for all prostate tissues examined, with cytoplasmic immunoreactivity observed in all prostate epithelial cells. In contrast to a diffuse cytoplasmic staining, luminal membrane

staining was found in PIN and primary and metastatic carcinoma tissues, with the most prominent membrane staining observed in poorly differentiated primary carcinomas and metastatic tissues. Based on the calculated stain indexes, PSMA was markedly overexpressed in the primary tissues with a high Gleason sum and in both metastatic lymph node and bone lesions. However, in contrast to primary tumors, the metastatic tissues demonstrated less staining heterogeneity. The reason for this apparent up-regulation with more uniform expression in the metastatic tissues is unknown. The effect of hormones on PSMA expression is currently being evaluated.

Although PSMA is an integral transmembrane protein, the cytoplasmic staining observed in prostate epithelium, especially in normal prostate tissues and well-differentiated tumors, could be explained by the location of the epitope in the cytoplasmic domain. Preliminary studies in our laboratory, using both light and electron immunomicroscopy, have demonstrated intracellular as well as membrane staining in cultured LNCaP cells.^{20,21} Immunoelectron microscopy showed Mab 7E11-C5.3 localization at the internal region of the plasma membrane, confirming the mapping of the antigenic epitope to the intracellular domain. Besides binding at the internal plasma membrane, Mab 7E11-C5.3 also localized to certain cytoplasmic organelles. Further studies are in progress to determine whether the cellular localization of PSMA observed in LNCaP cells also occurs in prostate tissues. In any event, strong evidence is presented that PSMA is largely expressed intracellularly (ie, intracellular organelles) and at the cytoplasmic face of the plasma membrane of LNCaP cells and prostate tissues.

The observed cytoplasmic staining pattern and localization raise the question of how the ^{111}In -labeled 7E11-C5.3 immunoconjugate (^{111}In -CYT-356) is able to image prostate cancer in vivo.^{8,9} Epitope-mapping experiments conducted in our laboratory have yet to demonstrate an epitope recognized by Mab 7E11-C5.3 in the extracellular domain of the PSMA glycoprotein (Troyer et al, unpublished results). As stated above, the only epitope recognized by Mab 7E11-C5.3 is located in the cytoplasmic domain. The sequence for this epitope is not found in the extracellular polypeptide region. It is quite possible, however, that the Mab binds to a similar but lower-affinity epitope expressed in the extracellular domain, thereby explaining successful imaging of the prostate cancer. Based on the amino acid sequence, there are numerous glycosylation sites available, suggesting that the extracellular peptide is heavily glycosylated. If this is true, then glycosylation may in fact mask the binding of Mab 7E11-C5.3 to these epitopes. Furthermore, carbohydrates are not part of the

epitope recognized by MAb 7E11-C5.3. Only the linear N-terminal peptide region is required for antibody binding. Based on these observations, the only mechanism for binding of the immunoconjugate to its antigenic target would be by binding to shed antigen in the intercellular spaces or passing through the plasma membrane to reach the epitope. The latter may be possible if the cells are undergoing apoptosis or necrosis. It is entirely possible that the intensely stained focal deposits in the cytoplasm of some malignant cells may represent apoptosis. This possibility is currently being explored. We know that PSMA is shed into prostatic fluid and is present in seminal plasma (Troyer et al, unpublished results), but we have not been able to confirm the initial observations⁷ that PSMA is also shed in serum. Further studies will be required to elucidate fully how the ¹¹¹In-CYT-356 immunoconjugate images prostate carcinomas.

Expression of PSMA appeared to correlate with tumor grade. When separate stain indexes were calculated for a random cohort of high-grade tumors (SI = 244) containing focal areas of low-grade tumor (SI = 59), the marked overexpression of PSMA in the high-grade areas became more clearly evident. In contrast, 90-100% of all prostate epithelial cells in these tissues intensely expressed PSA. In this study, PSMA expression did not correlate with pathologic stage. However, the majority of tumors examined were stage C disease. Because of the marked overexpression observed in poorly differentiated and metastatic prostate tumors, it might be expected that PSMA expression would correlate with the more aggressive and advanced stage D2 tumors. Additional studies will be required to determine whether this is the case.

Based on our initial observations of the intense membrane expression in the high-grade areas of primary tumors plus the overexpression in the metastatic tumor specimens, we postulated that this pattern of PSMA expression in the primary carcinomas would predict metastasis or tumor progression. However, this was not the case. Evaluation of the nodal status of 99 prostate cancer patients failed to show any correlation with the expression of PSMA in their primary carcinoma; nor was PSMA expression in the primary tumor predictive of extracapsular penetration or seminal vesicle invasion. Because most of the prostate carcinoma specimens were from patients who had had a radical prostatectomy, no attempt could be made in this series to determine the effects of radiation and hormone deprivation therapy on PSMA expression. However, we have noted in preliminary studies that hormone ablation therapy either has no effect on or up-regulates PSMA expression (Grob RM, Haley C, Newhall K, Schellhammer PF, Wright GL Jr, unpublished results). The effect of hormone ablation therapy on PSMA expression will be the subject of a separate report.

This study demonstrates the differential expression of PSMA in normal, benign, and malignant prostate tissues. The unexpectedly low expression in BPH tissues, as compared with normal and malignant prostate tissues, deserves further evaluation to determine the mechanism for the low expression and to exploit this observation as a possible means to differentiate BPH from CaP. We found that PSMA was overexpressed in the poorly differentiated and metastatic tumors. Although PSMA expression in the primary tumor was not predictive of metastatic disease, high expression appears to be

associated with the more aggressive prostate tumor, especially for hormone-refractory cancers. Because of the prominent intracellular location of the antigenic epitope, a mechanism to explain the clinical success of radiologic imaging with CYT-356 remains uncertain. Nevertheless, the restricted specificity, differential prostate tissue expression, and overexpression of PSMA in metastatic tissues support the continued study of this unique prostate tumor-associated biomarker for developing new strategies for the diagnosis and therapy of prostate cancer.

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References

1. Boring CC, Squires TT, Montgomery S. Cancer Statistics, 1994. CA 1994;44:7-26.
2. Osterling JE. Prostate-specific antigen: A critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. J Urol 1991;145:907-14.
3. Robbins AS. PSA and the detection of prostate cancer. JAMA 1994; 271:192-6.
4. Israeli RS, Powell CT, Fair WR, Heston DW. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. Cancer Res 1993;53:227-30.
5. Wright GL Jr, Feng Q, Liptford GB, Lopes D, Gilman SC. Characterization of a new prostate carcinoma-associated marker: 7E11-C5. Antibody, immunoconjugates, and radiopharmaceuticals 1991;3:39 (abs.).
6. Feng Q, Beckett ML, Kaladas P, Gilman S, Wright GL Jr. Purification and biochemical characterization of the 7E11-C5 prostate carcinoma-associated antigen. Proc Am Assoc Cancer Res 1991; 32(abs. 1418)239.
7. Horoszewicz JS, Kawinski E, Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostate cells and serum of prostatic cancer patients. Anticancer Res 1987;7:527-36.
8. Abdel-Nabi H, Wright GL Jr, Gullo JV, et al. Monoclonal antibodies and radioimmunoconjugates in the diagnosis and treatment of prostate cancer. Semin Urol 1992;10:45-54.
9. Wynant GE, Murphy GP, Horoszewicz JS, et al. Immunoscintigraphy of prostatic cancer: Preliminary results with ¹¹¹In-labeled monoclonal antibody 7E11-C5.3 (CYT-356). Prostate 1994;18:229-41.
10. Babaian RJ, Sayer J, Podoloff DA, Steelhammer LC, Bhadkamar VA, Gullo JV. Radioimmunosintigraphy of pelvic lymph nodes with ¹¹¹Indium-labeled monoclonal antibody CYT-356. J Urol 1994;152:1952-5.
11. Axelrod HR, Gilman SC, D'Aleo CJ, et al. Preclinical results and human immunohistochemical studies with 90Y-CYT-356: A new prostatic cancer therapeutic agent. J Urol 1992;147(abs. 596)361A.
12. Lopes AD, Davis WL, Posenstrauss MJ, Uveges AJ, Gilman SC. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from anti-prostate monoclonal antibody 7E11-C5. Cancer Res 1990;50:6423-9.
13. Starling JJ, Sieg SM, Beckett ML. Human prostate tissue antigens defined by murine monoclonal antibodies. Cancer Res 1986;46: 367-74.
14. Beckett ML, Liptford GB, Haley CL, Schellhammer PF, Wright GL Jr. Monoclonal antibody PD41 recognizes an antigen restricted to prostate adenocarcinomas. Cancer Res 1991;51:1326-33.
15. Wright GL Jr, Beckett ML, Liptford GB, Haley CL, Schellhammer PF. A novel prostate carcinoma-associated glycoprotein complex (PAC) recognized by monoclonal antibody TURP-27. Int J Cancer 1991;47:717-25.
16. Horoszewicz JS, Leong S, Xhu T, et al. The LNCaP cell line: A new

- model for studies on human prostatic carcinoma. *Prog Clin Biol Res* 1980;37:115-32.
17. Horoszewicz JS, Leong SS, Kawinski E, et al. LNCaP model of human prostatic carcinoma. *Cancer Res* 1983;43:1809-18.
 18. Israeli RS, Powell T, Corr JG, Fair WR, Heston DW. Expression of the prostate-specific membrane antigen. *Cancer Res* 1994;54:1807-11.
 19. Troyer JK, Feng Q, Beckett ML, Wright GL Jr. Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate-specific membrane antigens. *Urol Oncol* 1995;1:29-37.
 20. Troyer JK, Feng Q, Beckett ML, Morningstar MM, Wright GL Jr. Molecular characterization of the 7E11-C5 prostate tumor-associated antigen. *J Urol* 1993;149(abs. 482):333A.
 21. Troyer JK, Adam M, Wright GL Jr. Subcellular localization of the 7E11-C5 prostate specific antigen. *Proc Am Assoc Cancer Res* 1994;35(abs. 1688):383.

Molecular Foundations of Oncology

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Monoclonal Antibodies: They're More and Less Than You Think

Jeffrey Schlom

The title for this chapter was suggested by the editor of this book. After an initial period of shock, however, it began to make sense, both figuratively and literally. In the figurative sense, monoclonal antibody (mAb)-based applications (while very successful in diagnostics) have been very slow to be realized in cancer therapeutics. New concepts on how to employ existing mAbs and novel forms of mAbs, however, make many feel relatively optimistic about the future. Which brings me to the literal sense: the cloning and expression of mAb immunoglobulin (Ig) genes have now made feasible the addition of everything from human constant regions to toxins into mAb genes. Conversely, one can now subtract virtually everything from the anti-tumor mAb molecule except its binding site in the creation of single chain antigen binding proteins (SCAs). Thus, mAbs are indeed more and less than you probably think.

In this chapter, I will attempt to first define the various types of tumor-associated targets that mAbs may be directed against. Diagnostic applications of anti-tumor mAbs, while widespread, will be covered briefly and principally from the standpoint of aids to mAb-based therapeutic modalities. I will then discuss the varied modalities of mAb-based therapies, employing either unconjugated or conjugated mAbs; I will overview the advantages and shortcomings for each modality, and review previous and ongoing clinical trials and our current understanding of the parameters involved in these applications. The remainder of this chapter will deal with potential

future applications of mAbs. We are only now learning how to optimize the use of mAbs in combination with other biologic response modifiers and to develop optimal reagents for drug, toxin, and radionuclide conjugations to mAbs. Finally, emphasis will be placed on describing the basic principles of generating and utilizing recombinant chimerized (i.e., humanized) forms of mAbs and SCAs, as well as the technology for generating potentially novel mAbs with combinatorial libraries.

TARGETS FOR mAbs

There are several types of tumor-associated antigens (TAAs) that may act as targets for mAb-based diagnostics and therapeutics. The most common of these are the "oncofetal" antigens. These antigens are found in certain fetal tissues, one or several malignancies, and to varying degrees on certain normal adult tissues. The most commonly used mAbs to oncofetal antigens are mAbs 17-1A, KS 1-4, B72.3, OC125, L6, and an array of anti-CEA (carcinoembryonic antigen) mAbs (see Table 6.1 for listing of mAbs) (1-87). An interesting feature of many of these antigens is their pancarcinoma nature, i.e., their being expressed on a spectrum of carcinoma types. The second major class of targets are the so-called "differentiation" and "tissue-specific" antigens. The more well-defined mAbs in this class are the anti-TAC, CALLA, mAbs reactive with certain melanoma antigens, and mAbs to the human milk fat globule antigens. The third major class of

Table 6.1. Representative mAbs to human tumor antigens

Pancreaticoma	17-1A (11), 73-3 (2), 872-3 (3), CC49, CC83 (4), L6 (5), Mov-2 (6), AR-3 (7)
Breast	HMFG-1 -2 (8), 111D5 (9), DF-3 (10), MAM-6 (11), 115D8 (12), F36-22 (13), 24-17 (14), 323 A3 (15), MBr 1,2,3 (16), SP-2 (17)
Gastrointestinal	19-9 (18), HAG-1, -2 (19), ST-4-39 (20), DuPAN-2 (21), AR2-20, -28 (22), VII 23 (23), T84-66 (24), F33-37 (25), NP 1-4 (26), COL 1-15 (27), 791T-36 (28), 7E6A5 (29), Cora (30), D612 (31), CAA (32)
Prostate	F5 (33), F3, 2G7, 1C5 (34), anti-PAP (35), P62 (36), α -Pro 3 (37), 83-91 (38)
Bladder	T43 (39), T138 (39), HBA4 (40), J143 (39), 3G2-C6 (41)
Ovarian	OVB3 (42), OC125 (43), OM-1 (44), NB12123 (45), MOV-1 (6), MOV-18, -19 (46), OC133 (47), ID3, ID5 (48), OV-TL 3 (49)
Lung	Anti-LEU 7 (50), TFS-4 (51)
Small cell	KS 1-4, 1-7 (52), MOC-1 (53), LuCA-1, -4 (54), E10.15 (55), 43-9F (56), Po56 (57)
Non-small cell	96-5 (58), 48-7 (59), R24 (60), 14-18 (61), 11C64 (61), 140-240 (62), 9.2.27 (63), NR-ML-05 (64)
Melanoma	BF3, GE2 (65), CNT-2, -11, -8 (66), 3F8 (67)
Central nervous system tumors	anti-TAC (68), T101 (69), L17F12 (70), anti-T11, -T3 (71), anti-3A1 (72), anti-B1, -B4 (73), CALLA (73), anti-MO2, -MY9 (74), anti-id (75), anti-Lym-1, -2 (76), H65 (77), anti-CD22 (78), MB-1 (79)
Leukemias lymphomas	791T-36 (780)
Sarcomas	MBE6 (81), MCA series (82), R1-37 (83), 3B7 (84), L72 (85), CA27, CF29, JD39 (86); for others see Ref. 87 for review
Human mAbs	

Number in parenthesis indicates reference

targets are the growth factor receptors and oncogene products. Examples of these include mAbs to epidermal growth factor receptor (88) and the *c-erb-B2* oncogene product (89). One of the potential drawbacks to the use of mAbs to this type of gene product is their potential for a substantial degree of expression on cells of vital normal organs; however, quantitative differences in expression between malignant and normal tissues may in some cases make these antibodies potentially useful. mAbs to the antigen binding sites of anti-tumor antibodies, i.e., antiidiotype antibodies, offer yet another modality for cancer therapy by eliciting an active immune response to the tumor antigen in question. Such an approach has been demonstrated to be feasible in several animal tumor models (90, 91) and is now the subject of ongoing clinical trials with B cell lymphoma (76), gastrointestinal cancer (92), and melanoma (93). Finally, several groups have prepared native human mAbs by removing lymph nodes draining tumor from cancer patients and immortalizing them either by fusing with myeloma cells (murine or human) or via Epstein-Barr virus (EBV) transformation. Many of these mAbs (Table 6.1) are di-

rected against antigens, which are uncharacterized at this time; other have been shown to be directed against internal cellular proteins such as cytokeratin (see Ref. 94 for review).

Numerous mAbs to a spectrum of TAAs have now been developed and characterized. Table 6.1, which is only a partial listing of selected anti-tumor mAbs, categorizes over 100 potential therapeutics; extensive listings can be found in other review articles (95-98). Some of the more studied pancreaticoma mAbs are listed first. These mAbs have been shown to react with a wide range of carcinomas such as gastrointestinal, breast, gynecologic, and lung malignancies as well as to fetal tissues. Most of the selected mAbs listed under a particular carcinoma cell type, however, do not show restricted reactivity for only that tumor type. For example, the anti-breast cancer mAbs HMFG-1 and DF-3 will also show some reactivities to ovarian and other tumor types. It should also be pointed out that individual mAbs directed against the same antigen, such as the anti-CEA mAbs (Table 6.1) may differ greatly as to their range of reactivities to normal tissues, and other properties such as affinity: while only five groups of anti-CEA

mAbs are listed, dozens of anti-CEA mAbs have been generated and characterized (96, 99, 100).

As mentioned above, human mAbs have now been generated. These are reactive with antigens present in human tumors (Table 6.1) (87, 94); this has mostly been accomplished by fusing lymphocytes, biopsied from cancer patients, with myeloma cells. The rationale for the use of human mAbs is that (a) one may be able to identify novel antigens or determinants that are not immunogenic in mice by using conventional hybridoma technology, and (b) human mAbs will not elicit the immune response seen with murine Ig upon repeated administration in patients. This latter point, which will be discussed in detail below, may not be a major issue with the use of recombinant humanized mAbs, i.e., mAbs in which the variable region is of murine origin and the constant region is of human origin. To date, very few human mAbs, derived naturally by fusing human lymph nodes (see Table 6.1), have demonstrated the selective reactivity for tumor versus normal tissues to merit their being employed in clinical trials (101).

DIAGNOSTIC APPLICATIONS

There are numerous diagnostic and prognostic applications for antitumor mAbs, but these will be discussed here only in the context of adjuncts to therapeutic uses of mAbs. The first of these diagnostic applications involves the screening of body fluids such as serum, effusions, or urine for TAAs. In addition to identifying patients that have recurrent disease, as in the case of radioimmunoassays (RIAs) using mAbs to CEA, or the RIAs CA19-1, CA-125, CA 15-3, or CA 72-4, these serum assays may also be used to select patients whose tumors contain a particular antigen reactive with a given mAb (102, 103). Indeed, recent studies (104) have shown that patients with positive TAG-72 serum antigen levels are more likely to target the reactive B72.3 radiolabeled mAb. This is in contradiction to a belief (with very little hard data to back it up) that shed antigen in sera will bind administered mAb and thus make it inaccessible to tumor. In the end, each antigen system and particular mAb must be

evaluated independently with concern to this phenomenon.

mAbs have also gained widespread use in immunohistopathology and immunocytochemistry to define the presence of malignant cells, and for differential diagnosis among tumor types. Several studies with anti-tumor mAbs have shown a good correlation between the antigenic phenotype of primary and metastatic lesions. Consequently, immunohistochemical or immunocytochemical analysis of the primary tumor with a given mAb can potentially be used in studies to select patients for entry into therapeutic protocols employing that mAb.

PARAMETERS NOW DEFINED FOR mAb-DIRECTED THERAPY

Just a few years ago, virtually nothing was known about the parameters involved in optimizing mAb tumor targeting for diagnostic and/or therapeutic applications. As a consequence of numerous tumor targeting studies, some of the limitations and opportunities for mAb-based therapies are now being realized (Table 6.2). There are several properties of a mAb immunoglobulin molecule that will influence its usefulness as a therapeutic. In considering the use of an unconjugated mAb, the isotype becomes an important factor. Murine IgG_{2b} molecules appear most efficient in mediating antibody-dependent cellular cytotoxicity (ADCC) whereas murine IgG₁ molecules appear more efficient in complement mediated killing (CDC) (105-107). Recent studies with recombinant chimeric mAbs, however, have shown the human $\gamma 1$ isotype optimal in mediating ADCC with human effector cells (105).

The use of immunoglobulin fragments such as F(ab')₂, Fab, or Fv (only V_H and V_L domains) versus whole IgG should be considered when employing drug or radionuclide conjugated mAbs. mAb fragments have been shown to clear from the plasma pool at a much faster rate than whole IgG (108), a property particularly advantageous in the use of radiolabeled mAb with potential marrow toxicity of circulating unbound radiolabeled mAb. mAb fragments and Fvs should also have the advantage of a more rapid and even distribution through the tumor mass. Moreover,

Table 6.2. Parameters of mAb design and administration

Properties of tumor cells
Number of antigen molecules per cell surface
Number of cells expressing the relative antigen in the tumor mass
Fate of antigen-antibody complex
Stability on cell surface
Internalization
Capturing
Scavenging
Properties of the tumor mass
Size of the tumor mass
Degree of tumor vascularization
Degree of tumor mass infiltration and necrosis
Properties of immunoglobulins
Isotype of immunoglobulin (IgG subclasses or IgM)
Species of immunoglobulin (murine, human, or chimeric/recombinant)
Whole immunoglobulin or fragments (Fv, Fab, Fab')
Affinity and avidity
Clearance of mAb from blood (mAb metabolic stability)
Excretion
Reticuloendothelial system
Protocol design
Dose of mAb used
Route of inoculation of mAb (intravenous, intraperitoneal, intralymphatic, intradermal)
Dose fractionation of administered mAb
Other factors
Presence and reactivity of circulating antigen in the blood
Development of a human immune response to the administered mAb
To constant region
Anti-idiotypic
For drug or radionuclide conjugation
Ability of mAb to be conjugated with drug or radionuclide without loss of binding affinity
Specific activity
Number of drug or isotope molecules per Ig
Choice of drug or isotope
Linker technology
Use of marrow protectors such as IL-1 or colony-stimulating factors

immunoglobulin fragments have been shown to elicit less of a human antiimmunoglobulin response than whole IgG (109-112). A disadvantage in the use of fragments, however, is that in most animal model studies a lower percent injected dose is delivered to the tumor compared to that of intact IgG. This appears most likely due to the fact that Fab' is breaking up into Fab fragments in vivo,

thus losing bivalency and, consequently, affinity. Furthermore, Fab fragments will clear the blood at such a rapid rate that they may diminish time for accumulation of the fragments in the tumor.

A wide range of mAb doses have been used in the clinical trials completed to date. Gram quantities of unconjugated mAb 17-1A (113) have been administered with no evident toxicity. Moreover, as little as 0.1 mg of radiolabeled mAb (114) has been shown to efficiently localize tumor. To date, no major a priori toxicity has been demonstrated that can be attributed to the use of mAbs alone. However, when mAbs are conjugated to particular drugs, toxins, or radionuclides, toxicities attributed to the conjugates have been documented (115, 116). Little or no toxicity has been attributed to the development of the HAMA (human antimurine antibody) response; in those few cases in which an anaphylactoid reaction has occurred, standard treatment has led to its immediate cessation. The major disadvantage in the development of the HAMA response is the consequent rapid clearance of the administered mAb, which diminishes its ability to target tumor.

It has been demonstrated that, following one dose of mAb, approximately 50% of patients develop HAMA; this increases to approximately 90% in patients receiving multiple (three or more) doses of mAb (109, 110). Thus, in reality, in virtually all the previously reported human therapy trials in nonimmunosuppressed patients in which multiple administration of murine mAbs were used, only the first and/or perhaps the second mAb administration were efficiently reaching the tumor site. It is unrealistic to assume that just one or two administrations of a given anticancer therapeutic could be effective. As will be discussed below, the use of recombinant/chimeric mAbs could potentially circumvent this situation. Following numerous injections of recombinant/chimeric mAbs, however, some antiimmunoglobulin response may result. This could be directed toward the idiotypic region, i.e., the binding site of the hypervariable region or to an allotypic region. The immune response to certain murine sequences could be further reduced or eliminated by their substitution

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with human sequence, i.e., the immunoglobulin molecule could be "humanized" as in the case of the mAb CAMPATH-1 (117) and anti-TAC (118). Another potential way to overcome this problem is the use of Fc (119) molecules, also termed single chain antigen binding proteins (SCA), which consist of just the combining sites of the V_H and V_L molecules, held together by a linker. A third way to potentially address this problem is to create a family of antibodies with the same target antigen but with different idiotype determinants; these could be used sequentially as a way of circumventing the human antiidiotypic response. Although the potential for the development of an antiidiotypic response following numerous administrations of recombinant chimeric mAbs is real, it may not be as pronounced as that which has been seen when administering murine mAbs. The reason for this may be that the murine Fc is actually acting as a carrier and thus enhancing the immune response to the idiotype determinants. Thus, if the human Fc is less immunogenic, a diminished antiidiotypic response may also be manifested.

The size of the tumor mass will also likely be an important consideration in mAb-based therapy. It has been postulated that larger tumor masses may exert vascular pressure in a manner as to make some parts of the tumor mass inaccessible to mAb (120). This may well be the case involving large tumor masses, but has not been the case with smaller tumors in experimental models (121). Another potential problem is that large tumor masses may contain so much antigen that they may act as a "sink" and bind all available mAb leaving other parts of the tumor or other tumor masses in that patient without sufficient mAb for therapeutic efficacy. Large tumor masses may also have more necrotic centers with less vascularity, making them less accessible to mAb.

There are several additional factors that one must also consider in the use of drug, toxin or radionuclide conjugated mAbs. These include: (a) the ability of the mAb to be conjugated and still maintain its ability to bind target antigen with the same affinity (Some mAbs, for example, contain tyrosines in their binding sites and cannot be radiolabeled with ^{125}I by conventional techniques.); (b) the spe-

cific activity, i.e., how many molecules of drug, toxin, or radionuclide should be conjugated to the immunoglobulin molecule for maximum killing potential without reduction in binding ability; (c) method of linkage of drug, toxin, or radionuclide; and (d) immunogenicity of the toxin. This latter phenomenon has proven to be a problem in more than one situation.

One of the areas of active investigation at the present time is the development of linker technology to efficiently couple drugs and radionuclides to immunoglobulin molecules. This will be discussed later in the area of drug conjugation (122). New bifunctional chelates are now being developed to more efficiently keep radioactive heavy metal killer isotopes such as ^{90}Y in the mAb complex (123). One area that has received little attention to date is the metabolism and catabolism of the immunoglobulin. For example, in the use of conjugated mAbs, what is the ultimate fate of the complex? Will it be taken up by organs such as the liver and/or kidney and lead to unwanted toxicities or be rapidly eliminated from the body with minimal toxicity? Modifications of recombinant immunoglobulins in terms of size and glycosylation can be employed to alter some metabolic patterns, if deemed necessary.

Thus, as a result of what one may refer to as the first round of mAb-based protocols, several limitations in the use of currently available mAbs have become evident. These include: (a) the development of HAMA; (b) antigenic heterogeneity; (c) the lower percentage of the injected dose per gram that can be delivered to the tumor; (d) difficulty in diffusion of immunoglobulin through large tumor masses; (e) in selected cases, free antigen in circulation, which may bind administered mAb; (f) toxicities associated with conjugated drugs, toxins, and radionuclides when coupled to the mAb or in the case of their dissociation; and (g) lack of absolute specificity of the mAb. At this time, it seems unlikely that binding to some normal tissues, such as normal colon, will necessarily negate the use of many mAbs. Although each of these limitations cannot be minimized, many can now be overcome in practice and in theory, as will be discussed below.

Several advantages to mAb-based ther-

apy, however, have now also become apparent. Most importantly, the mAb has been demonstrated to have the advantage of selective reactivity for the tumor antigen. Whereas no mAb has thus far been developed with absolute specificity for tumor versus normal tissues, it has clearly been demonstrated, using radiolabeled mAbs, that antitumor mAbs can target tumors with tumor to normal tissue ratios of 3:1 to up to 20:1 or more (124). Moreover, the toxicity associated with the administration of mAbs in several thousand patients has been minimal. Other advantages in the use of mAbs are that one can actually develop a "therapeutic index" for a given patient by preadministration of a diagnostic dose, and/or help select patients for therapy using a given mAb by analysis of biopsy specimens for the presence of high levels of target antigen using immunohistochemical techniques. Finally, one can modify mAbs to suit specific purposes. This has previously been confined to the use of immunoglobulin fragments; however, with recombinant technologies utilized in the cloning of immunoglobulin genes, a vast array of modifications are now possible.

MAB TUMOR TARGETING TRIALS (PRE-PHASE I)

Dozens of institutions are now employing radiolabeled mAbs for tumor targeting. The more extensively used mAbs to date are anti-CEA (24, 125-128), B72.3 (124, 129-132), 17-1A (133), OC125 (43), and HMFG (134), for targeting of carcinoma, anti-p97 and anti-HMW antigen for melanoma (135), and Lym-1 (76) for targeting of lymphoma. Other mAbs are also being evaluated (136-141). A recent review of the literature (Larson S, unpublished data) indicates that over 3000 patients have been enrolled in diagnostic tumor targeting trials using radiolabeled mAbs: in these studies, six different radionuclides have been employed and 25 different tumor types targeted. For example, in a recent study (142), 509 patients from 11 nuclear medicine centers were administered Flab[®] fragments of an anti-CEA mAb: 75% of tumor lesions were visualized, and occult disease was detected in 30% of patients.

Recent multicenter trials using ¹¹¹In-

labeled B72.3 (also termed CYT-103) (143) demonstrated 74% positive antibody images confirmed at surgery (143). When compared with the results of computerized tomography (CT) scans, the mAb scans showed greater sensitivity (73% versus 52%) for tumor lesions, and mAb scans affected management in 26% of patients evaluated. Promising results have also been obtained using ¹²⁵Ie-labeled mAb NR-ML-05 to target melanoma lesions (64): 77% of noncutaneous lesions were detected. In studies using a radioconjugate of mAb 3F8, 90% of neuroblastoma lesions have been accurately identified (Larson S, Cheung NKV, unpublished data) (144). Diagnostic studies with several other radiolabeled mAbs have also been extremely successful. To date, Product License Applications for at least three different mAbs are being considered by the Food and Drug Administration for use in this modality.

In addition to the use of radiolabeled mAbs to detect occult carcinoma and aid in staging, this modality also has therapeutic implications: the use of a given mAb-radionuclide conjugate in low doses (for diagnostic purposes via γ scanning), may provide a "therapeutic index" for that particular mAb, whether the mAb is given coupled to a high energy killer isotope or drug or used unconjugated. Thus, these types of trials are now referred to as "Pre-Phase I" trials. It is this author's belief that any new mAb being considered for clinical trials—either used unconjugated or conjugated to drugs, toxins, or radionuclides—should first be administered in low doses as a radiolabeled product to help define: (a) its ability to target tumors at different sites, (b) any unsuspected normal tissue targeting, and (c) pharmacokinetics of blood, cavity, and whole body clearance.

The type of information that can be gained by these types of Pre-Phase I trials can be exemplified in studies carried out with mAb B72.3. Whereas these types of studies have also been carried out with other mAbs, B72.3 will be used here as an example because of familiarity and its use at numerous institutions (145). Using *in vitro* assays, mAb B72.3 was first shown to be reactive with a range of human carcinomas (gastrointestinal, gynecologic, non-small cell lung, and mammary), and not, or weakly, reactive with a

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range of normal adult tissues (146) with the exception of secretory phase endometrium (147) and transitional colonic epithelium adjacent to carcinoma (148, 149). Preclinical studies in mice using ^{125}I -radiolabeled B72.3 then demonstrated good tumor localization (150). Radiolabeled mAb B72.3 was thus administered to colorectal cancer patients for a quantitative evaluation of its reactivity with tumor lesions versus a wide range of normal tissues. These studies included patients with metastatic colorectal carcinoma that involved the surgical resection of metastatic colorectal cancer lesions plus adjacent "normal" tissues for staging and therapeutic purposes. Thus, γ scanning followed by a comprehensive direct examination of tumor and a variety of normal tissues could be achieved. Approximately 7 days before surgery, patients received ^{125}I -labeled mAb B72.3 IgG. Patients were scanned with a γ camera at various times until surgery. At surgery, suspected carcinoma lesions and selected normal tissues were removed. Specimens from all biopsies were immediately weighed and placed in a γ counter to determine counts per minute per gram; biopsy specimens were subsequently analyzed for the percentage of tumor cells of total cells present and the percentage of TAG-72 antigen-positive cells.

In the initial study, 27 patients received ^{125}I -labeled B72.3 IgG. Positive γ scans (confirmed at surgery) were observed in 14 of 27 patients and in 10 of 20 patients in which direct examination of tissues were available. There was no effect of IgG dose, milliCurie amount, or specific activity on whether a γ scan was positive or negative. γ scans accurately identified tumor lesions in the liver, bone, orbit, rectum, colon, caecum, and pelvis and diffusely in the peritoneal cavity. No toxicity was observed. Perhaps the most important part of these studies, however, revolved around analysis of a radiolocalization index (RI). The RI value is defined as the ratio of the uptake of ^{125}I -labeled mAb in tumor to that of histologically confirmed normal tissue on a per gram basis: average values (counts per minute per gram) from biopsy of normal liver and/or intestinal tissue of each patient were normalized to 1.0. RI values of ≥ 3.0 were arbitrarily considered as "positive" for these studies. At least one tumor lesion in 17 of 20

of the patients studied had an RI of ≥ 3.0 . In eight of these patients, all 50 tumor lesions biopsied had RI values of ≥ 3.0 . Five of the patients studied displayed 26 lesions with RI values of ≥ 10.0 . In total, 99 of 142 (70%) carcinoma lesions biopsied displayed RI values of ≥ 3.0 (114, 124) (Table 6.3). Of 210 histologically confirmed normal tissues biopsied, 198 showed negative RI values, i.e., less than 3. In 12 patients, all apparently normal tissue biopsies had a negative RI. Twelve biopsy specimens from eight patients showed RI values of ≥ 3.0 . In all but two cases, these tissues were immediately adjacent to carcinoma or draining carcinoma; in two cases, positive RI values were seen in spleen biopsies, which were apparently due to the presence of antigen-antibody immune complexes. These studies thus demonstrate the type of information that can be obtained about a given mAb in Pre-Phase I trials.

Intracavitary Administration

An alternative approach in the use of mAbs for diagnostic or therapeutic purposes involves intracavitary administration. mAbs administered directly into a cavity or body site may localize tumors in that cavity more efficiently than intravenously (i.v.) administered mAb. Studies have been conducted to: (a) determine the feasibility of intraperitoneal (i.p.) administration of radiolabeled mAb for tumor localization or therapy, (b) determine the specificity of tumor localization by the concomitant i.p. administration of ^{125}I -mAb and an isotype matched control ^{125}I -labeled mAb, (c) compare tumor localization of i.v. versus i.p. administered mAb by the simultaneous administration of ^{125}I -mAb i.v. and the same ^{125}I -mAb i.p., and (d) define the pharmacokinetics of plasma clearance of both i.p. and i.v. administered radiolabeled mAb. Studies of this type have been carried out in ovarian and colorectal carcinoma patients with mAbs HMFG (141) and B72.3 (129).

In a recent study (129), patients were administered ^{125}I -labeled mAb B72.3 murine IgG i.p. and scanned with a γ camera at various time points prior to surgery. γ scans of 7 of 10 patients showed clearly discernible concentrations of radiolabeled mAb in tumor. A representative positive γ scan is shown

Table 6.3. *Antitumor mAb B72.3 Binding to Human Carcinoma Lesions*

Tissue	Antitumor mAb B72.3		Control mAb BL-3
	Number of Patients	Number of Lesions	
Colon	1	1	1
Stomach	1	1	1
Small Intestine	1	1	1
Uterus	1	1	1
Liver	1	1	1
Bladder	1	1	1
Prostate	1	1	1
Spleen	1	1	1
Heart	1	1	1
Brain	1	1	1
Total	10	10	10

RI = Ratio of tumor to normal tissue uptake. RI = 1.0 indicates equal uptake of both mAbs in tumor and normal tissue. RI > 1.0 indicates greater uptake of B72.3 than BL-3 in tumor tissue.

The antitumor mAb B72.3 was administered intravenously (i.v.) at a dose of 100 µg/kg body weight. The control mAb BL-3 was administered intravenously (i.v.) at a dose of 100 µg/kg body weight.

Antitumor mAb B72.3 was administered intravenously (i.v.) at a dose of 100 µg/kg body weight. The control mAb BL-3 was administered intravenously (i.v.) at a dose of 100 µg/kg body weight.

in Figure 6.1: this patient was negative for tumor when analyzed via CT scans. Three of the initial 10 patients studied that were positive for mAb localization via γ scanning (areas confirmed as tumor at surgery) were negative for tumor via CT scanning and x-ray studies. Lesions as small as approximately 1.5 cm in diameter were clearly defined via γ scans. The RI values of biopsy specimens were determined. Using an RI of ≥ 3 , which was arbitrarily chosen as a "positive" radiolabeled uptake, 83 of 112 (74%) of carcinoma lesions showed positive values. Of the 95 histologically confirmed normal tissues biopsied, all but one demonstrated RI values of ≤ 3 .

Dual mAb Administration Studies

To determine if the mAb radiolocalization observed was specific (in this case with the i.p. administered ^{125}I -B72.3 IgG), studies were conducted in which patients received concomitant infusions (administered i.p.) of ^{125}I -B72.3 IgG and ^{125}I -labeled control mAb BL-3 IgG. mAb BL-3 uptake was negative in most carcinoma lesions but showed RI values of ≥ 3 in some. In 31 of 39 lesions, the B72.3 BL-3 ratios were greater than 2:1, with ratios of greater than 10:1 in the majority of cases: ratios of greater than 100:1, moreover, were

observed for many lesions. Thus, while these results demonstrate the specificity of the antitumor mAb B72.3 binding, they do point out that "nonspecific" binding of irrelevant mAb to carcinoma lesions may and does indeed occur at times.

Studies were also conducted to determine the relative efficacies of i.p. versus i.v. administered mAb to localize tumor lesions (129). To achieve this goal, patients were concomitantly administered ^{125}I -labeled B72.3 i.p. and ^{125}I -labeled B72.3 i.v. In some patients, intracavitary administered mAb has been shown to target peritoneal tumor better than systemically administered mAb. Not surprisingly, however, these same dual route mAb administration studies demonstrated that systemically administered mAb can target hematologically borne metastases, such as liver metastasis, with greater efficiency than mAb administered into the peritoneal cavity. Another conclusion from this study is that one may plan future trials to involve the use of a therapeutic dose of mAb in which some is given systemically and some is given via a direct or intracavitary route. Figure 6.2, exemplifies that a diagnostic dose (10 mCi/1 mg) and a subsequent therapeutic dose (100 mCi/10 mg) of the same mAb can demonstrate similar tumor targeting. These types

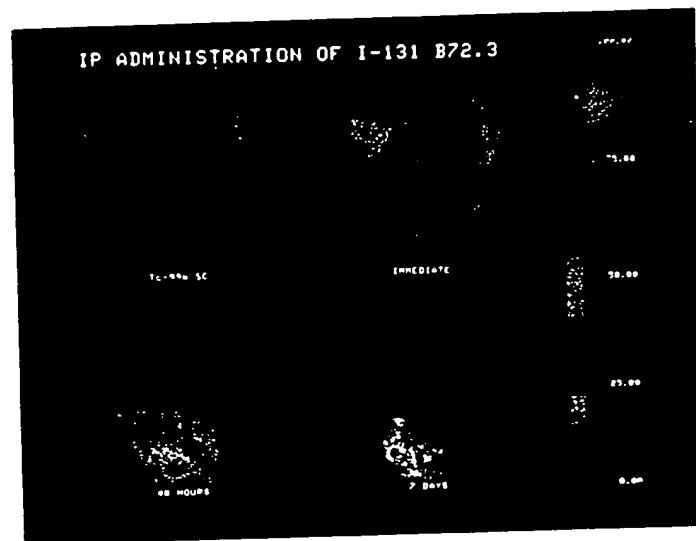


Figure 6.1. Intrapertoneal administration of ^{131}I -labeled mAb B72.3 in a colorectal carcinoma patient. All views are of the peritoneal cavity (from diaphragm to bladder). Upper left, Tc-99m scan; upper right, immediately following mAb administration; lower left, 48 hours post mAb administration in which mAb is seen to begin to localize tumor; lower right, mAb clearly visualized small tumor lesions, which were confirmed at surgery. This patient was negative for tumor by CT scans (132) (From Raubitschek A, Colcher D, Lastoria S, et al. Intrapertoneally administered radiolabeled monoclonal antibodies in patients with peritoneal carcinomatosis [Abstract]. Second Conference on Radioimmunodetection and Radioimmunotherapy of Cancer, Princeton, NJ 1988).

of Pre-Phase I "diagnostic" studies carried out with several mAbs have consequently formed the basis for therapeutic studies, many of which are now ongoing (see below).

Pharmacokinetics of mAb Clearance

One of the parameters that must be considered in diagnostic or therapeutic mAb applications is mAb pharmacokinetics. The data in Figure 6.3 demonstrate a typical clearance curve for an i.v. administered murine IgG in patients. Note that approximately 50% of the injected dose appeared in the plasma at day 2 with approximately 10–15% of the injected dose found in plasma at day 7 post mAb administration. In contrast, no more than 30% of the injected dose of i.p. administered mAb appeared in the plasma at any point in time, with peak values being obtained at days 2 to 3 (Fig. 6.3). One of the

objectives of recombinant Igs and their constructs will be to alter mAb pharmacokinetics as desired.

INTRAOPERATIVE PROBE mAb-DIRECTED SURGERY

A novel diagnostic therapeutic use of mAbs involves the employment of a hand-held γ detecting probe used at surgery to detect occult tumor. This probe has now been used in several hundred patients at numerous centers in conjunction with ^{125}I -labeled mAbs such as 17-1A, anti-CEA, and B72.3. In one study involving colorectal carcinoma patients, the intraoperative probe localized the ^{125}I -B72.3 in 83% of sites and altered the surgical approach in 26% of patients with recurrent colorectal cancer (151). In a more recent study, 14 patients with carcinoma of the breast confirmed at biopsy received

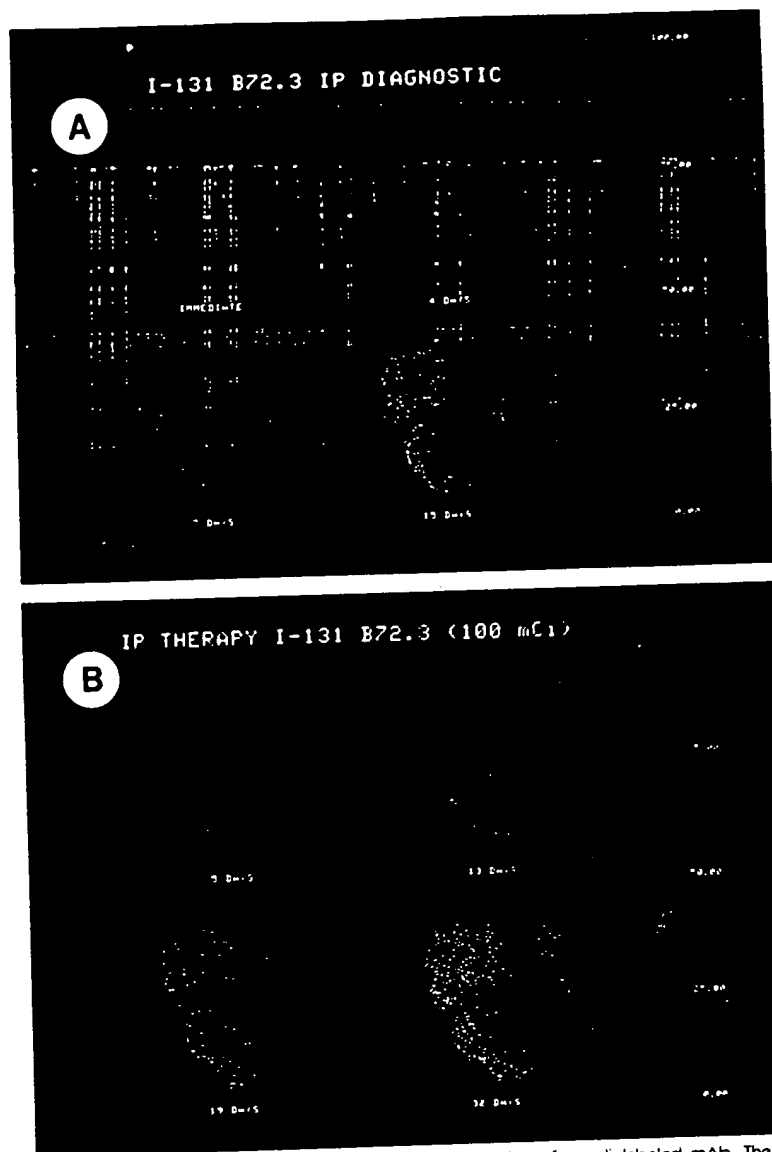


Figure 6.2. Comparative diagnostic and therapeutic tumor targeting of a radiolabeled mAb. The top panel represents a diagnostic dose (approximately 10 mCi/mg) of ^{131}I -B72.3 administered intraperitoneally (IP) to a patient with colorectal cancer. All views (top and bottom) are of the peritoneal cavity. Note tumor localization at days 4 through 15 (all confirmed via CT scan). This patient was thus selected for administration of a therapeutic dose (approximately 100 mCi/10 mg) of the same mAb (bottom). Note good tumor targeting at the times indicated through day 32 (132). (From Raubitschek A, Colcher D, Lastone S, et al. Intraperitoneally administered radiolabeled monoclonal antibodies in patients with peritoneal carcinomatosis [Abstract]. Second Conference on Radioimmunodetection and Radioimmunotherapy of Cancer. Princeton, NJ, 1988.)

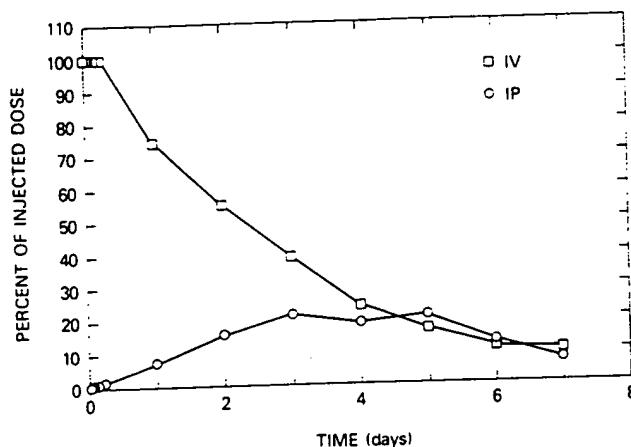


Figure 6.3. Comparative plasma clearance of mAb IgG after administration both i.v. and i.p. in patients with colorectal cancer. Patients were injected i.v. with ^{125}I -B72.3 IgG and i.p. with ^{125}I -B72.3 IgG. Plasma samples were drawn at various times. The open circles denote the mAb clearance levels for patients receiving i.p. administered mAb. The open squares denote the mAb plasma clearance levels from patient receiving i.v. administered mAb (129). IV, intravenous; IP, intraperitoneal.

^{125}I -labeled mAb i.v. prior to further exploration: the γ detecting probe, used for this application externally, was able to identify residual, subclinical, and multicentric carcinoma of the breast in some cases (152).

SPECTRUM OF mAb-BASED THERAPY MODALITIES

Unconjugated mAbs

There are several therapeutic approaches that one may employ using a given mAb—from the unconjugated form to elicit antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or to activate the idiotype pathway to elicit an antidiotype host immune response, to the use of drug, toxin, or radionuclide conjugated reagents. Many of these approaches have proved successful to one degree or another in experimental model systems (61) and all are currently being tested in clinical trials. In 1980, a preliminary serotherapy trial was reported using mAb 89 directed against a lymphoma-associated antigen (153). In vitro studies indicated that this mAb could mediate CDC but not ADCC. Up to 1.5 g of mAb

was administered per patient, and a transient decrease in the number of circulating tumor cells was noted. Clinical trials were also conducted with mAb J-5, which is reactive with the common acute lymphoblastic leukemia antigen (CALLA). These studies (154) showed a rapid clearance in circulating blasts, but not all leukemic cells were cleared; the remaining cells appeared to be resistant to further serotherapy. An early clinical trial (70) using a mAb to a normal T cell differentiation antigen in a patient with T cell leukemia also showed a rapid but transient fall in white blood cell count. Antigenic modulation by leukemic cells was also observed.

One of the most promising approaches to the therapy of T cell malignancies involves the use of the mAb anti-TAC (155) which binds to the p55 chain of the human interleukin-2 (IL-2) receptor and has been shown to inhibit proliferation of T cells by blocking IL-2 binding (see page 138). A clinical trial involving the i.v. administration of anti-TAC in nine patients with the human T-cell lymphotropic virus I (HTLV-I)-induced adult T cell leukemia resulted in three patients with transient mixed, partial, or complete remissions lasting from 1 to more than 8 months post therapy (156).

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Currently, clinical trials are ongoing involving unmodified anti-TAC, 125 I-conjugated anti-TAC, and truncated *Pseudomonas* toxin conjugates of anti-TAC, and with recombinant chimeric "humanized" anti-TAC (118).

Phase I and II trials have now been carried out in gastrointestinal patients with mAb 17-1A (157) and melanoma patients with mAb R24 (60). Some partial responses were reported, but virtually all patients developed HAMA. At the present time, unconjugated mAbs 17-1A (157, 158), L72 (85), B72.3 (132), 3F8 (67), 96.5 (59), R24 (60), T101 (69, 159), L17F12 (70), and other antilymphoma mAbs (154, 160) are in clinical trials. Several of these trials are now showing some objective clinical responses. The limitation of this general approach, however, is that not all mAbs may have cell killing properties via ADCC or CDC mechanisms and that all tumor cells must express the target antigen. The advantage of this approach is minimal toxicity and ease of mAb production and handling.

An alternative approach in the use of unconjugated mAbs in tumor therapy is to use antitumor mAbs (Ab1s) and/or the mAbs (Ab2) directed against the idiotype (binding site) region of those mAbs to activate the idiotype network. The network theory of idiotype roles in the regulation of the immune response postulates that an antigen can elicit the production of the first antibody (Ab1). Ab1, in turn, stimulates Ab2, which can elicit the Ab3, etc. Initially, this model of complementary idiotype interactions was thought to be an open-ended network. Further studies have demonstrated a more limited idiotype network.

Those antiidiotypic antibodies that specifically bind to the Ab1 paratope can thus inhibit Ab1 binding to antigen and are thought to conformationally mimic the antigenic epitope. These Ab2, which can possess the "internal image" of the antigen, could be used as immunogen to induce protective responses; these Ab2 could also be utilized to break the tolerance of otherwise nonimmunogenic molecules. Numerous antitumor experimental studies have now been carried out using mAbs to activate the idiotype (Id) network.

In 1985, the development of a goat polyclonal Ab2 that reacted specifically with the

idiotype of the mAb 17-1A was reported. When this Ab2 was injected into rabbits and mice, it elicited an Ab3 response that bound the same antigen as mAb 17-1A. In a xenogeneic system, mouse mAb2s were developed to a rat mAb1 recognizing an oncofetal bladder carcinoma antigen. These Abs demonstrated the ability to induce specific cellular-mediated immune responses in rodents. This same group also generated Ab2 to the p97 antigen of human melanoma (161). These Ab2 were shown to induce an Ab3 response in syngeneic or allogeneic mice. A mouse lymphoma model (L1210-GZL) has been used to study the idiotype cascades in mice (90). An Ab1 was used that recognizes a TAA that cross-reacts with the GP52 of the mouse mammary tumor virus. Two of the antiidiotypic mAbs could induce an antigen-specific humoral response, delayed-type hypersensitivity (DTH) response, and cytotoxic T-lymphocyte (CTL) responses. However, only one of these mAbs could offer protection from tumor growth. Other antiidiotype (anti-Id) experimental systems are currently under investigation.

Studies with antiidiotypic antibodies in clinical trials have focused on the B cell malignancies, which should be optimal targets for Ab2 therapy. First, B cell malignancies have been demonstrated to originate from a single clone so that all malignant B cells should express an identical surface immunoglobulin (162-165). Therefore, these molecules are true examples of tumor-specific molecules and are optimal targets of mAb therapy (166). Second, B cell malignancies are more readily exposed to the vascular and reticular effector systems, as compared to solid tumor systems. In 1980, the first studies were reported of patient treatment with antiidiotypic antibody (167). Here, a patient with chronic lymphatic leukemia (CLL) received an infusion of a rudimentary preparation of the globular fraction of a sheep antiserum. The individual underwent a transient reduction in circulating leukemic cells accompanied by considerable toxicity. Two years later, a mouse monoclonal IgG_{2b} antiidiotypic antibody was utilized to treat a patient with follicular lymphoma (75). This patient, who had failed chemotherapy, was infused with increasing doses of mAb2 over a period of 4 weeks. Remarkably, a complete remission was observed and

the individual remains disease free without continued therapy. In 1985, mAb2s were utilized to treat 10 patients who also had advanced stages of disease (168). Unfortunately, the results were not as profound as those of the previous study. Five of 10 patients exhibited significant reductions in tumor size (45–100%). Unfortunately, tumors usually returned within a period of 1–6 months. Interestingly, the relapsing tumor no longer reacted with the idiotype, indicating either modulation of Ig or an idiotypic variation.

Alternative V κ gene rearrangements were first characterized in a murine cell lymphoma (169, 170) and have been hypothesized as an explanation for observed heterogeneity. Recently, immunoprecipitation studies in a human B lymphoma cell line also demonstrated major alterations in λ light chains (171). A major disadvantage of this kind of B cell lymphoma therapy is that treatment may have to be "custom-made" for each individual's lymphoma (172). This process has been streamlined by directly immunizing mice with patient lymphoma cells versus purifying the idiotype and then immunizing. Recently, "shared" idiotypic determinants among patients have been identified. Thus, it is possible that a finite panel of anti-Id mAbs can be drawn upon for this modality (173).

The combination of antiidiotypic antibody therapy with interferon- α (IFN- α) has recently been reported (174). In this study, 12 patients received Ab2 and IFN- α ; there were two complete responses and several partial responses. The tumor cells remaining were Id negative, indicating a strong antitumor effect to Id-positive cells, resulting in a strong selection for the Id-negative cells.

In terms of solid tumors, sera from patients immunized with mAb 17-1A (Ab1) were shown (175) to contain antiidiotypic antibody that was able to inhibit Ab1 from binding to antigen. It was concluded from this data that the patients who developed the "internal image" Ab2 were those that improved clinically and developed long remissions. Furthermore, the appearance of Ab3 was demonstrated in culture supernatants of peripheral blood monocytes from five patients treated with mAb1 (176). Thus, it was concluded that idiotypic cascades do exist in humans immunized with antitumor immu-

noglobulin. It has also been shown that Ab2 responses exist in patients receiving the anti-CEA mAb NP-2 (177). Ten patients with advanced gastrointestinal cancer were infused with mAb three times weekly. Five of these patients developed combining site-specific antiidiotype antibodies. However, no clinical response was observed. Clinical studies involving the administration of an Ab2 mAb, produced from an antimelanoma mAb, are currently in progress, with some partial responses reported (178).

These clinical studies all support the existence of a network cascade of immune interactions. However, these should be regarded only as initial studies to determine if there will be a role for this modality in the elimination of tumor.

Drug and Toxin mAb Conjugates

The coupling of mAbs to drugs has the advantage of taking a wide range of agents with known antitumor activity and pharmacology and enhancing their relative tumor uptake via the mAb. The most commonly cited theoretical limitation to the potential usefulness of mAb drug or toxin conjugates as effective oncolytic agents is the necessity of the mAb conjugate to internalize for cytotoxic activity. The observation that some solid tumor membrane antigens are stable cell surface components suggests that a subset of mAb drug conjugates will be ineffective against these target antigens. The phenomenon of antigen heterogeneity is also an important consideration since the antigen-negative tumor cell will not bind the immunconjugate and therefore may escape death. The above limitations may not apply, however, if the chemical bond utilized to link the drug to the immunoglobulin is sufficiently labile to permit dissociation of the cytotoxic agent at the tumor cell periphery followed by transport into the antigen-positive or nearby antigen-negative cell. For example, the chemistry has been described (179) whereby a hydrazide derivative of a vinca alkaloid was coupled to immunoglobulins, via aldehyde groups formed by periodate oxidation of the oligosaccharide side chains, which may be an example of such a labile bond. mAb KS 1:4 vinca conjugates directed against a stable membrane antigen

were shown to have potent antitumor activity in vivo even though a number of in vitro assays failed to detect rapid internalization of the antibody-antigen complex (122). Several clinical trials with drug- and toxin-conjugated mAbs have been initiated (115, 116, 180-182). For example, a trial in 22 melanoma patients with a murine mAb conjugated to ricin A chain immunotoxin demonstrated localization of mAb and A chain to sites of metastatic disease, but also demonstrated a range of toxicities related to the dose of immunotoxin (182). A Phase I trial with mAb 260F9, which targets a 55,000 molecular weight antigen expressed on mammary carcinoma cells, coupled to ricin A chain, was suspended after four patients developed neurologic toxicity believed to be associated with the mAb (116). mAb 791T/36, which recognizes a 72,000 molecular weight antigen on the surface of the colon carcinoma cells, was coupled with ricin A chain and used in a Phase I trial in 17 patients with metastatic colon cancer (183). The side effects observed included a composite of symptoms thought to be generic to ricin A chain immunotoxins. By 10-20 days after therapy, most patients developed IgM and IgG antibodies against both the ricin A chain and the Ig portion of the immunotoxin; an anticomplementing site antibody response was also seen. Mixed tumor regression was seen in 5 of 17 patients.

Clinical trials are currently under way with second and third generation immunotoxin conjugates. mAb anti-CD22 has been coupled to a deglycosylated form of ricin A chain and employed in Phase I/II trials in patients with refractory B cell leukemia/lymphoma (Vitetta E. Thorpe PE, unpublished data). Of 18 patients studied, toxicities were transient, and a 60% mixed or partial response was observed. Recent studies employing a mAb anti-B4 "blocked" ricin conjugate (in which ricin cell binding sites are blocked) have also been promising in Phase I trials of CD19 antigen-positive B cell malignancies (Nadler L, unpublished data). In a trial involving 25 patients using five bolus administrations of mAb conjugate per patient, one complete response (18 months duration) and two partial and 10 transient responses were observed. When a constant infusion of the same immunoconjugate was

used, of 15 patients, one complete, three partial, and four transient responses were observed. Transient hepatocellular toxicity was observed.

It seems quite likely that the construction of active mAb drug conjugates will have to take into account the biology of the antibody-antigen complex (i.e., stable versus internalized), the lability of the chemical bond used to link the drug to the antibody, and the mechanism of action, potency, toxicity, and immunogenicity of the drug or toxin itself.

Radionuclide mAb Conjugates

There are both advantages and disadvantages in the use of radiolabeled mAb conjugates. One of the major advantages is that several isotopes can kill at several cell diameters; therefore, one can envision a radiolabeled mAb conjugate binding to an antigen-positive cell and killing adjacent antigen-negative cells. Thus, the use of a radiolabeled mAb conjugate may be another way of overcoming tumor cell antigenic heterogeneity. Another advantage in the use of radiolabeled mAb conjugates is that the antigen-radionuclide complex need not internalize to kill the tumor cell. There are also many radionuclides available with different energies and half-lives that can be used to suit a given therapeutic circumstance.

Therapeutic trials with several radioconjugated mAbs are now in progress. Pioneering studies with ^{131}I -labeled anti-ferritin polyclonal antibodies in hepatoma patients reported several clinical responses (184). More recently (127), ^{131}I -anti-CEA polyclonal antibodies, given in combination with external radiation and therapy in patients with primary nonresectable cholangiocarcinoma, were reported to give a 26% partial response rate. Studies (137) with ^{131}I -labeled mAb HMFG2 given intrapleurally in a limited number of patients showed partial or complete responses with minimal toxicity. Reports (134, 141) using this same mAb given intraperitoneally showed its ability to limit accumulation of antigen-positive malignant ascites. Radiolabeled mAbs have also recently been administered intrathecally in patients with leptomeningeal tumors. The choice of anti-

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body varied depending on the immunophenotype of the tumor (136, 139). Therapy was generally well tolerated with minimal acute toxicity. Four of five patients achieved an objective response, which was sustained for 7 months to 2 years. Several clinical trials are now in progress using ^{131}I - and/or ^{90}Y -labeled mAbs such as B72.3 (native and chimeric), anti-Lym-1, anti-TAC, and several anti-CEA mAbs.

A recent clinical trial involving ^{131}I -labeled mAb anti-Lym-1 use in B cell malignancies showed some complete and some partial responses (DeNardo S, unpublished data). Other institutions, using anti-Lym-1 conjugated to ^{131}I , however, found only a few partial responses, and no response if extranodal disease was present (Salehan S, unpublished data). The reason for these discrepancies is unknown at this time.

In recent trials involving the use of mAb MB-1 conjugated to ^{131}I in patients with B cell lymphoma, whose marrow had been cryopreserved prior to therapy, some promising results have been obtained (Eary JF, unpublished data). Of 22 patients entered into the study, only those patients who displayed good mAb scans using low ^{131}I -mAb doses were entered into the therapy arm. Of the eight patients treated, most had a complete or partial remission, with durations greater than 15 months. Little toxicity was noted. Patients received up to 600 mCi followed by successful marrow replacement. Using another approach, ^{90}Y -anti-Id mAb is being administered to patients with B cell malignancy (Parker B, unpublished data). Studies employing the use of mAb T101- ^{90}Y conjugate in CD5-positive B and T cell malignancies have also been initiated. Of the initial seven patients treated, three partial responses have been observed (Raubitschek A, unpublished data). Ongoing studies with ^{131}I -labeled mAb 3F8 in neuroblastoma patients have also resulted in four clearly measurable responses in the first nine patients entered (Larson S, Cheung NKV, unpublished data).

Some of the disadvantages in the use of radiolabeled mAb conjugates involve difficulties in preparation, storage, and handling of radioactive material, as well as real or perceived safety considerations of medical care personnel, and in some cases, special

patient rooms for a limited amount of time. Unbound circulating radiolabeled antibody can result in the dose-limiting marrow toxicity. The use of new chelates (123, 185) designed to hold the radionuclide more tightly in the antibody complex may prove an important advance for this modality. Finally, as mentioned above, the use of a given antibody-radioconjugate in low diagnostic doses followed by γ camera scanning, may provide a "therapeutic index" for selected patients to go on to subsequent therapeutic doses.

INNOVATIONS IN mAb-BASED THERAPIES

Up Regulation of Target Antigens

Virtually every property of a tumor cell population has been shown to demonstrate some degree of heterogeneity or modulation, either between different tumor masses or among cells of a given tumor mass; this phenomenon has been demonstrated for hormone and growth receptors, drug resistance, oncogene product expression, etc. Expression of some tumor-associated antigens is no exception. It is unclear at this time, however, whether antigenic heterogeneity reflects the emergence of a new stable antigen-phenotype or a temporary modulation of expression of an antigen on the preexisting phenotype. Indeed, much evidence is now emerging that what has been viewed as "antigenic heterogeneity" may actually be antigenic modulation. Factors such as spatial configuration (186), or exposure to biologic response modifiers (187, 188) have been shown to have the ability to alter the expression of some tumor antigens. One approach is thus to better understand the phenomenon of antigenic modulation and to find ways of manipulating it to one's advantage.

The use of biologic response modifiers to up regulate TAAs on the tumor cell surface is currently being pursued in several laboratories, with the goal that these studies will lead to enhanced mAb binding to the tumor cell. For example, it has been demonstrated that certain recombinant IFNs, such as α (clone A), β -ser and γ , can actually up regulate the expression of several TAAs, such as some melanoma antigens, and carcinoma

antigens such as CEA and TAG-72 (189) on the cell surface. It has been shown that the concentrations of IFN required to induce this phenomenon can be well below cytostatic and cytotoxic doses (189). The mechanism for the phenomenon is not clearly understood at this time, but it has recently been shown that recombinant IFN can up regulate steady state message of the CEA gene (190). It has also been shown (191) that the TAAs CEA and TAG-72 can be up regulated on approximately 80% of fresh biopsy specimens of effusions of a variety of carcinomas and not up regulated on benign effusions and a variety of normal cell lines. When recombinant IFN- α was injected into mice bearing human tumor xenografts, it was shown to up regulate antigen expression and enhance the binding of administered radiolabeled antitumor mAb (192). It has also been shown that IFN can enhance tumor targeting of radiolabeled mAb in melanoma patients (193). Recent studies have demonstrated, in ovarian and gastrointestinal cancer patients, that relatively low doses of i.p. administered recombinant IFN- γ can increase the expression of the TAG-72 antigen on carcinoma cells in effusions within 24 hours after administration (194) (see Fig. 6.4). These findings may thus ultimately prove extremely important in enhancing both diagnostic and therapeutic modalities of mAb-based therapies.

Synergism Between mAbs and Other Biologic Response Modifiers

Several experimental studies in vitro and in animal models have now demonstrated that mAbs may act synergistically with effector cells to mediate tumor cell cytotoxicity, i.e., ADCC. For example, using a syngeneic mouse model, it has been shown (195) that an antitumor antigen mAb can work synergistically with IL-2 to activate endogenous murine LAK cells to more efficiently eliminate liver metastases. Recently, it has been shown (31, 61) that some antitumor mAbs can act synergistically with human LAK cells to more efficiently mediate killing of human tumor cells than either component alone. It has also been shown that antitumor mAbs may be chemically cross-linked with anti-Fc receptor mAbs to form heteroantibodies that can more efficiently

mediate T cell killing of human tumor cell populations; this phenomenon has been detailed in a recent review (196).

Numerous studies are now being undertaken to use mAbs in combination with other biological response modifiers; for example, the use of lymphokines such as IL-1 or colony-stimulating factors in conjunction with therapeutic doses of mAbs coupled to drugs or killer isotopes could well reduce marrow toxicity (197). Finally, with the development and use of recombinant chimeric mAbs, one can now directly introduce sequences for linkers of drugs or toxins, the toxins themselves, or biological response modifiers directly into the immunoglobulin molecule for more efficient delivery to tumor masses.

Second and Third Generation mAbs

As outlined in Table 6.1, mAbs to a range of tumor antigens have been generated, some of which are now in clinical trials. There is no a priori reason to assume, however, that the first mAb to a given tumor antigen is the best in terms of specificity, affinity, isotype, etc. This has been seen with the HMFG antigens (8) and with the TAG-72 antigen. For example, the original B72.3 mAb was used to purify the reactive TAG-72 antigen. This antigen was then employed as immunogen to produce a second generation of mAbs (4). As a result of extensive testing and selection, some of these mAbs have been shown to have higher affinities, to more efficiently bind human tumor xenografts than the original B72.3 and are more efficient in preclinical therapy experiments. The structure of many tumor antigens recognized by mAbs have now been elucidated, and the genes coding for these tumor-associated antigens have been, or are in the process of, being defined. This may lead to the preparation of synthetic peptides that can be used as immunogens and thus a new generation of mAbs to as yet undefined determinants. The use of combinations of mAbs should not be ruled out in future therapy studies. For example, mAbs to CEA and the TAG-72 antigen have been shown to be complementary in their reactivities to human carcinoma populations (198).

It should also be pointed out that the

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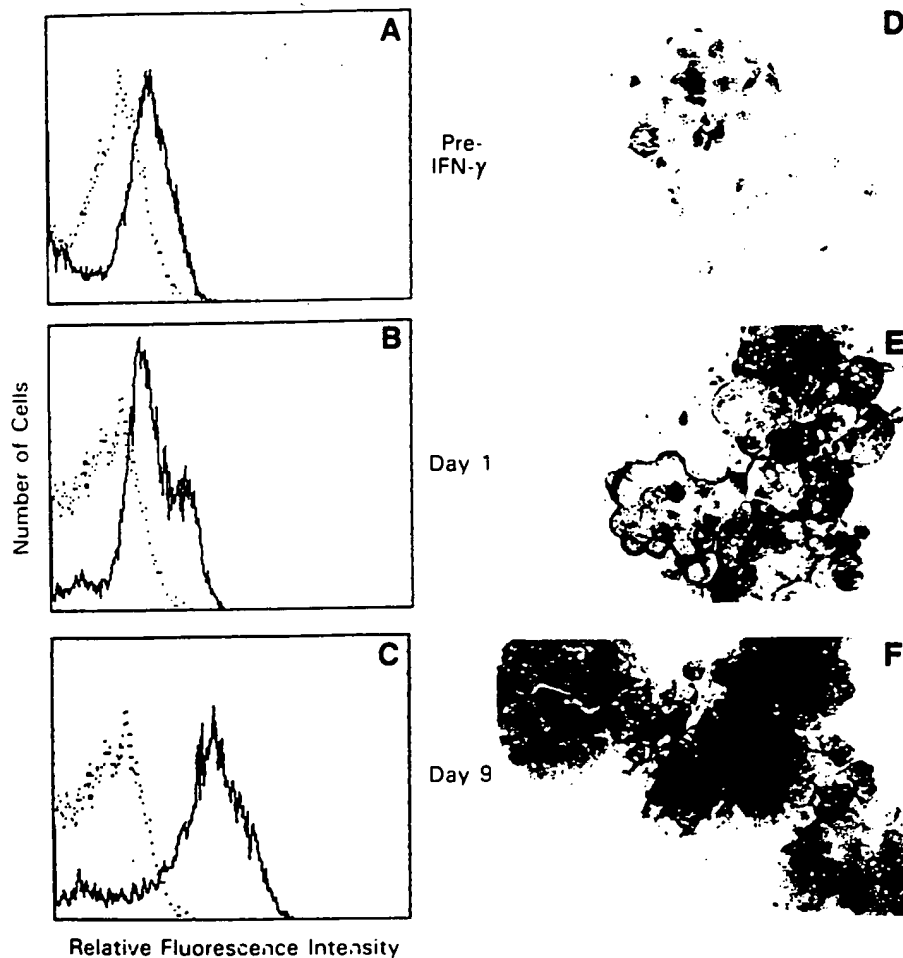


Figure 6.4. The use of recombinant interferon to up regulate tumor antigen expression. Changes induced in B72.3 binding to human ovarian carcinoma cells isolated from adenocarcinomatous ascites before and after the patient received two successive weekly i.p. administrations of IFN- γ (0.1 million units). Prior to the initial i.p. treatment with 0.1 million units IFN- γ , ascites was removed and the constitutive TAG-72 expression was measured by B72.3 binding using flow cytometry (A) and immunocytochemistry (D). B and E illustrate the alterations in B72.3 reactivity to carcinoma cells isolated 24 hours after i.p. IFN- γ treatment. The patient was given a second i.p. treatment with 0.1 million units of IFN- γ , and B72.3 binding was analyzed by flow cytometry (C) and immunocytochemical staining (F) 48 hours later (i.e., day 9) (194). Reddish brown stain indicates tumor antigen-positive cells. (From Greiner JW, Guadagni F, Goldstein D, et al: Preclinical and phase I/II clinical studies demonstrating that interferon-gamma (IFN- γ) augments the level of tumor associated glycoprotein-72 (TAG-72) expression on human ovarian carcinoma cells [Abstract]. 1989 Annual Meeting of the International Society for Interferon Research, Florence, Italy, 1989.)

generation of antitumor mAbs has led to the isolation, by mAb affinity chromatography, of novel tumor-associated antigens. These antigens can now be better characterized and purified and be employed as reagents for active immunotherapy of cancer patients. One example of this is the vaccinia p97 melanoma antigen construct, which has proven successful in preventing the transplant of tumor cells containing this antigen in preclinical animal studies (199). The vaccinia p97 construct has also been shown to elicit immune responses to the p97 antigen in nonhuman primates.

DOSE FRACTIONATION PROTOCOLS

As discussed in detail elsewhere in this chapter, one of the major drawbacks in mAb-based therapies to date has been the development of the HAMA response after just one or two mAb administrations. This response has thus abrogated the effectiveness of second and subsequent mAb administrations by clearing mAb via immune complexing and thus reducing the percentage of mAb that reaches the tumor site. These problems may be vastly reduced by the development of recombinant/chimeric mAbs, humanized mAbs, or SCAs. Furthermore, recombinant/chimeric mAbs, humanized mAbs, or SCAs make possible the employment of efficient dose fractionation protocols.

Very few systematic studies have been carried out, however, to determine if dose fractionation protocols involving mAbs will actually enhance antitumor activity and/or reduce toxicity. A recent study was carried out to test this principle. mAb B72.3 was radiolabeled with ^{131}I and administered to athymic mice bearing the LS-174T human colon carcinoma xenograft to determine if mAb dose fractionation had any advantage in tumor therapy. The LS-174T xenograft, in which only approximately 30–60% tumor cells express the B72.3 reactive TAG-72 antigen, was used to reflect the antigenic heterogeneity often seen in patient biopsy specimens. In contrast to a single 600- μCi dose of ^{131}I -B72.3 where a few animals had reduced tumor growth, but 50% of the animals died from toxicity (Fig. 6.5), the administration of two 300- μCi doses of B72.3 (total of 600 μCi) re-

duced or eliminated tumor growth in 90% of mice, with only 10% of the animals dying from marrow toxicity. Three-dose fractionations even permitted escalation of the dose to 900 μCi resulting in even more efficient tumor reduction or elimination and minimal toxicity (Fig. 6.5). Tumors that had escaped mAb therapy were analyzed for expression of the TAG-72 antigen and were shown to have the same antigenic phenotype as untreated tumors. The use of an isotype-matched control mAb revealed a nonspecific component to tumor growth retardation, but the use of the specific B72.3 demonstrated a much greater therapeutic effect. These studies thus demonstrate (121) the potential advantage of an efficient dose fractionation of a radiolabeled mAb for tumor therapy. It is anticipated that this concept can now be applied in radioimmunotherapeutic clinical trials with the development and use of recombinant/chimeric mAbs and modified constructs.

GENETICALLY ENGINEERED AND CHIMERIZED ANTIBODIES

In the opinion of some, the ability to genetically clone, alter, and express Ig genes is to mAb research and employment, as great a quantum leap in immunology as was the transition from the use of polyclonal sera to the advent of hybridoma technology and the use of monoclonal Igs. mAbs gave us the ability to define the rules and parameters of Ig-based diagnostics and therapeutics and basic Ig functions. Now, recombinant Igs and their altered forms give us the ability to overcome many of the shortcomings of existing molecules, optimize functions, and deploy new functions by genetically engineering into molecules. A review of recombinant/chimeric mAbs has recently been written (200). This section will contain some of the more relevant points made previously and delineate how this new technology may be of direct consequence to cancer therapy applications.

The potential for the use of genetically engineered mAbs in the management of cancer is enormous. Perhaps the most immediate advantage of recombinant/chimeric mAbs will be the reduction of the HAMA response. It has been demonstrated that the vast majority of this response is directed toward the

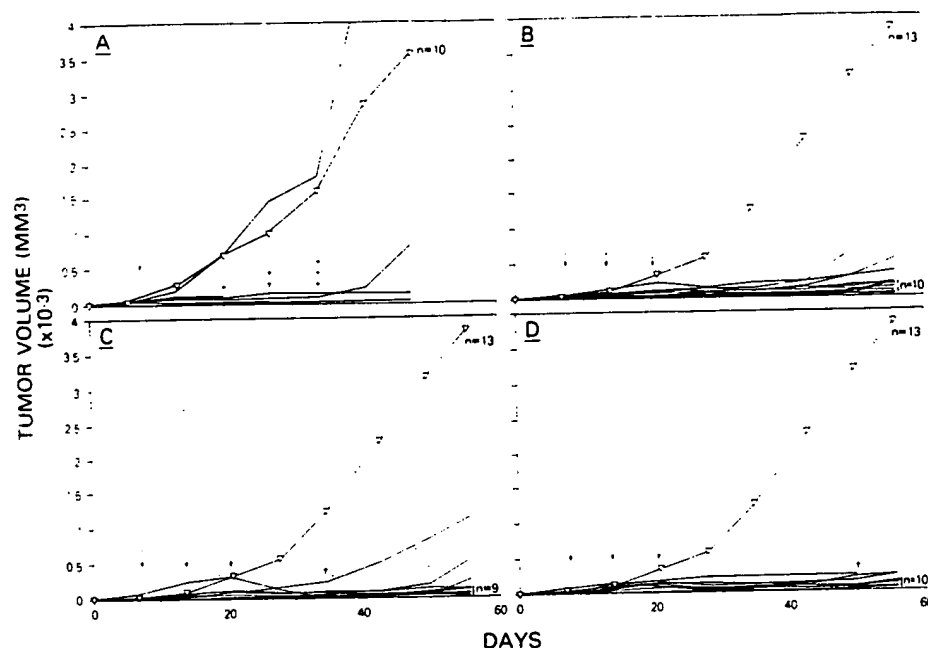


Figure 6.5. Advantage of a dose fractionation protocol. Effect of administration of dose fractionated ^{125}I -labeled B72.3 IgG on the growth of the LS-174T colon carcinoma xenograft in athymic mice. Each line represents the growth of the tumor in an individual mouse. Arrows represent the time(s) of administration post tumor implantation. Crosses represent the death of a mouse. **A** shows the effect of a single administration of 600 μCi of ^{125}I -B72.3. Open triangles in all panels are the results of 10 mice that received an equal dose of unlabeled B72.3. **B**, **C**, and **D** represent the effect of dose fractionation as follows: **B**, 200 μCi of ^{125}I -B72.3 \times 3 for a total of 600 μCi ; **C**, 250 μCi of ^{125}I -B72.3 \times 3 for a total of 750 μCi ; **D**, 300 μCi of ^{125}I -B72.3 \times 3 for a total of 900 μCi (121).

murine Fc region of the Ig molecule (111, 112). The major consequence of the HAMA response is the rapid clearance of the administered mAb due to antibody-antibody complexing; an example of this is shown in Figure 6.6. The mAb clearance may be so rapid as to prevent the vast majority of the mAb from reaching the tumor site. Thus, in reality, in virtually all the previously reported human therapy trials using multiple administrations of murine mAbs, only the first and/or perhaps the second mAb administrations were optimally reaching the tumor site. It is unrealistic to assume that just one or two administrations of a given anticancer therapeutic could be effective. Thus, the preparation and administration of recombi-

nant/chimeric or humanized anti-tumor mAbs may eliminate or greatly diminish this problem. Following injections of recombinant-chimeric mAbs, however, some anti-Ig response may result. This could be directed to certain structures within the murine variable region and perhaps directed toward the idiotype region, i.e., binding site of the hypervariable region.

There are numerous ways in which antibodies can be modified. Antibodies are formed by polypeptide chains held together by noncovalent forces and disulfide bridges (Fig. 6.7). A pair of identical L and H chains form a bilaterally symmetric structure. The polypeptide chains fold into globular domains separated by short peptide segments:

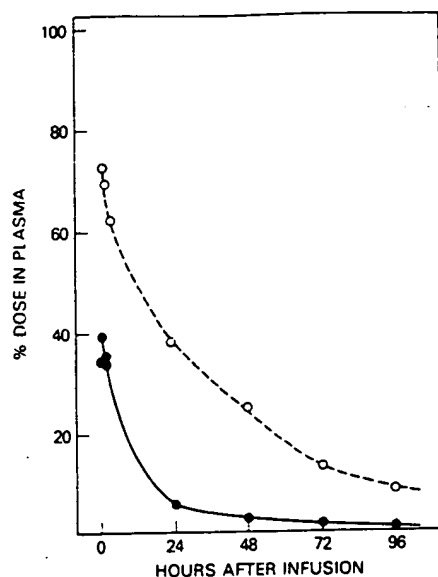


Figure 6.6. Serum clearance of protein-bound radioactivity following injection of a patient with ^{125}I -labeled mAb 9.2.27 is shown. The percentage of the injection dose retained in plasma after the first (open circles) and second (closed circles) mAb administration is shown (230). Note the rapid serum clearance due to the development of HAMA.

the H chain has four or five domains depending on the isotype, and the L chain has two. The N-terminal portion of each chain constitutes the variable region (V_H , V_L). A V_H - V_L pair carries the antigen combining site and contributes to antibody specificity; this section of the antibody is designated the Fv region. The remainder of the molecule constitutes the constant (C) region, the region of the molecule responsible for effector functions such as Fc receptor binding, complement fixation, catabolism and placental transport. Immunoglobulins with different C regions and therefore of differing isotypes (in humans they are IgM, IgD, IgG1-4, IgA1, IgA2, and IgE) may exhibit different biological properties. In some isotypes, a hinge region separates C_H1 and C_H2 and provides the molecule with segmental flexibility. The enzyme papain cleaves near the hinge to generate the Fab and Fc portions of the antibody molecule.

Antibodies are glycoproteins with the carbohydrate content varying among different isotypes. One uniform feature of all IgG isotypes is that each H chain contains a single carbohydrate moiety at Asn-298 in C_H2 . The carbohydrate is not only important in maintaining the structure of the IgG but also is essential for some effector functions.

According to Greek mythology, a chimera is a monster with a lion's head, a goat's body, and a dragon's tail. Chimera has now come to characterize things put together from diverse gene segments not normally found associated (200). Chimeric antibody genes are genes assembled from diverse gene segments not normally found associated. Chimeric immunoglobulins can be intraspecies, where variable and constant regions are from the same species or interspecies, where, for example, the variable region is of mouse origin and the constant region is of human origin. Chimeric Igs can include antigen-binding specificities joined to non-Ig protein structures.

The ability to genetically manipulate Ig genes and then express these altered genes enables one to produce mAbs that have improved properties when compared to the existing hybridoma antibodies. Table 6.4 contains a list of some of the possible alterations of Ig genes and their potential biologic consequence. To produce genetically engineered antibody molecules, three prerequisites must be fulfilled: (a) a recipient cell must be available that produces functional antibody molecules, (b) there must be a means to efficiently introduce DNA into the recipient cells, and (c) expression vectors must be available that both permit the expression of the antibody genes and the isolation of the rare recipient cells expressing the introduced genetic information (200).

Antibodies are multichain proteins with heavy and light chain polypeptides held together by disulfide bonds. Proper assembly must take place to produce a functional molecule. To make a complete functional antibody molecule, the recipient cell must be able to assemble, glycosylate, and secrete the multichain antibody molecule. Recombinant Ig molecules have now successfully been produced in bacteria, yeast, a range of mammalian cells (both B cells and nonhemato-

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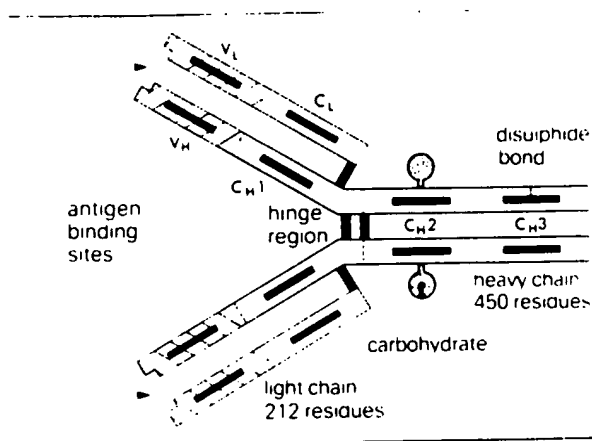


Figure 6.7. The basic structure of IgG. The amino-terminal end is characterized by sequence variability (V) in both the heavy (H) and light (L) chains, which are referred to as the V_H and V_L regions, respectively. The rest of the molecule has a relatively constant (C) structure. The constant portion of the light chain is termed the C_L region. The constant portion of the heavy chain is further divided into three structurally discrete regions, C_{H1} , C_{H2} , and C_{H3} . These globular regions, which are stabilized by intrachain disulfide bonds, are referred to as domain. The sites at which the antibody binds antigen are located in the variable domains. The hinge region is between the C_{H1} and C_{H2} domains in most Igs. Flexibility in this area permits variation in the distance between the two antigen binding sites, allowing them to operate independently. Carbohydrate moieties are attached to the C_{H2} domains (231).

poietic cells), and most recently in plants (200, 201). To date, most recombinant/chimeric antitumor mAbs have been produced in mammalian cells. Even under optimal conditions, gene transfection into eukaryotic cells is an inefficient procedure with only rare recipient cells becoming stably transfected. Thus, vectors should be used that contain biochemically selectable markers that permit the selection of the rare stably transfected cell lines. The most commonly used vectors to date have been based on the pSV2 vectors (202, 203). These vectors contain three essential elements: (a) a plasmid origin of replication, (b) a gene encoding a biochemically selectable phenotype in bacteria, and (c) a gene encoding a biochemically selectable marker in eukaryotic cells (Fig. 6.8). To create antibody molecules, the two genes encoding heavy and light chains must be transfected into the same cell, and both polypeptides must be synthesized and assembled.

A number of murine mAbs that recog-

nize TAAs have demonstrated potential for both targeting tumors and for tumor therapy, either unconjugated via ADCC, CDC, or conjugated to anticancer agents or radio-nuclides. The utility of these antibodies has been limited in part by the development of a HAMA response and/or failure of the antibody to possess the desired biologic properties such as serum clearance pharmacokinetics and perhaps tumor penetration. Mouse/human chimeric antibodies comprised of variable regions derived from murine hybridomas and human constant regions will presumably be superior for administration to humans due to their reduced immunogenicity, altered pharmacokinetics, and ability to function more effectively with human effector cells. To date, Ig genes encoding a number of murine mAbs with reactivity to tumor-associated antigens have been cloned and expressed (Table 6.5). Chimeric antibodies have been prepared utilizing human constant region genes joined with variable regions derived from the re-

Table 6.4. Advantages and Disadvantages of Chimeric Antibodies and Consequences

Advantage	Disadvantage
1. Reduced toxicity to normal cells One to murine Fc region replaced Antigen binding site (variable region) is human IgG or IgE	a. Enhance effector cell- and complement-mediated tumor lysis b. Reduce immunogenicity
2. Smaller Other in pediatric Immunogenic reactions Preparation of Fc: SSCAs	a. Alter pharmacokinetics and blood clearance and whole body clearance b. Alter mAb metabolism patterns (e.g., kidney and liver uptake) c. Reduce immunogenicity
3. Improved Addition of pediatric Fc substitution Manipulation of sites in constant or variable region	a. Alter pharmacokinetics and blood clearance and whole body clearance b. Alter mAb metabolism patterns (e.g., kidney and liver uptake) c. Alter effector cell functions
4. Higher	Enhance mAb affinity
5. Binding site	Enhance mAb affinity and binding specificity
6. Insertion of drugs and drug response modifiers at binding sites for those molecules	A more stable immunocjugate resulting in more efficient tumor cell killing-less toxicity

spective mAbs. In all cases, the resulting chimeric proteins retained the specific binding characteristics of the murine mAbs. An example of this is seen in Figure 6.9 in which the recombinant (containing murine Fc) and recombinant chimeric (containing human Fc) B72.3 compete completely for the binding of native B72.3 to the TAG-72 antigen. In pre-clinical radiolocalization studies, chimeric antibodies have been shown to efficiently localize human xenograft tumors in athymic mice (204, 216). An alternate approach to chimerization has been to "humanize" the antibody by the insertion of hypervariable regions from the heavy and light chain domains of a rat mAb reactive with a human lymphocyte antigen, CAMPATH-1, into a human IgG1 (κ) antibody. A recent report (217) described the treatment of two non-Hodgkin's lymphoma patients with a fully reshaped (chimeric) antibody CAMPATH-1. Tumor reduction was reported in both patients without the development of a serologically detectable antiimmunoglobulin response. Recently, the anti-TAC mAb has also been humanized (118).

Chimeric antibodies have been shown to exhibit biological properties appropriate for their respective human Ig subclasses (105, 112). Chimeric antibodies consisting of the murine variable region derived from the mAb

17-1A coupled with human γ1, γ2, γ3, and γ4 constant regions have been evaluated for their abilities to mediate ADCC of human tumor-cell targets in the presence of human and murine effector cells and compared with the parental murine IgG_{2a} antibody. Monocytes and macrophages utilized chimeric IgG₁, and to a lesser degree IgG₁ and IgG₂, to mediate tumor cell lysis in ADCC assays.

In some instances, the substitution of human constant regions has resulted in enhanced antitumor activity or has imparted biological activity to a nonfunctional antibody possessing desirable binding specificity. For example, both murine (IgG_{2a}) and chimeric (IgG₁) forms of mAb L6 exhibit identical affinities for the carcinoma-associated L6 antigen, and both mediate CDC. However, chimeric L6 mediates ADCC in the presence of human effector cells at a concentration 100 times lower than that required for native murine L6. Additionally, chimeric L6, but not murine L6, lysed a melanoma cell line expressing small amounts of L6 antigen. mAb B72.3, which is a murine IgG₁, has weak ADCC activity. The recombinant/chimeric B72.3 with a human γ1 constant region has recently been shown to mediate ADCC of human ovarian carcinoma cells in combination with human LAK cells (218).

In the first study involving a recombi-

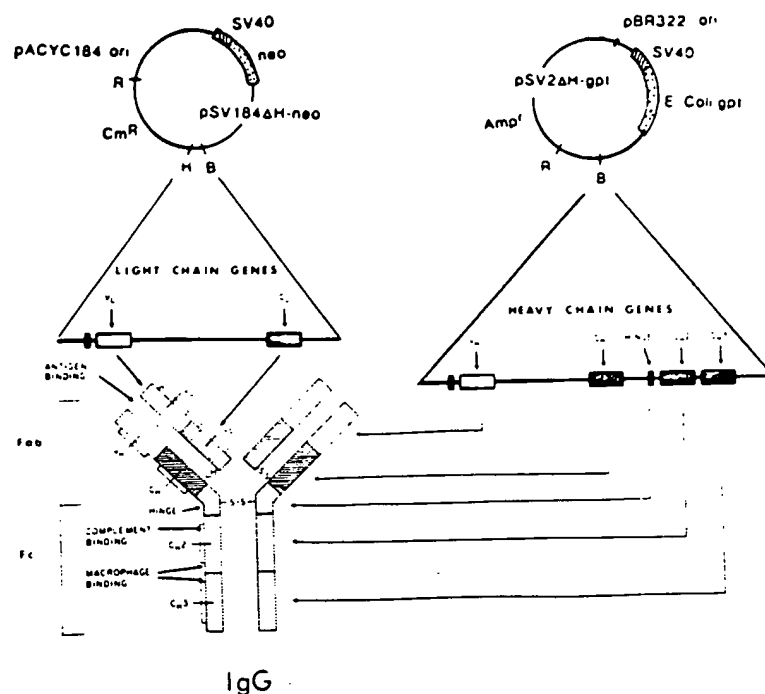


Figure 6.8. Vectors used to deliver DNA for expression of immunoglobulin genes. The pSV2 vectors contain the replication origin (ori) from the plasmid pBR322 and the β -lactamase gene providing resistance to ampicillin (amp^r); the pSV184 vectors contain the replication origin from pACYC184 and the chloramphenicol acetyltransferase gene providing resistance to chloramphenicol (cm^r) (pBR322 and pACYC184 are compatible plasmids). In this example, the pSV2 vector contains the heavy chain gene and the eukaryotic transcription unit expressing the *Eco-gpt* gene providing resistance to mycophenolic acid. The pSV184 vector contains the light chain gene and the *neo* gene in the eukaryotic transcription unit, providing resistance to G418. The exon structure of both the light and heavy chain genes is depicted. Arrows indicate the correspondence between the DNA segments and the different domains of the Ig polypeptide chain they encode. The hydrophobic leader sequence of both heavy and light chains is removed after synthesis and is not present in the mature immunoglobulin molecule (222).

nant/chimeric mAb (112). a mouse/human chimeric construct composed of murine mAb 17-1A and the constant region of human IgG₁ was administered to colon cancer patients. Patients received either single infusions of 10 or 40 mg of recombinant/chimeric 17-1A, or three infusions of 10 or 40 mg at 2-week intervals. The pharmacokinetics of recombinant/chimeric mAb serum clearance were similar at both dose levels and at second and third mAb infusions, thus indicating the absence of any functional anti-Ig responses. The plasma clearance curves of the recombinant

chimeric 17-1A were best fit by a two-compartment model having a mean of $t_{1/2}$ of 17.5 hours and a mean $B_{1/2}$ of 100.5 hours. Only one of 10 patients appeared to demonstrate an anti-Ig response following three mAb administrations: this response first appeared, however, at day 63 post mAb administration, was of relatively low titer, and was directed toward the murine variable region of the chimera (112). The patients in this study had no toxic or allergic reactions. Thus, the recombinant/chimeric form of 17-1A had a longer circulation time and appeared to be

Table 6.5. Recombination of murine and human mAbs

mAb	Target Antigen	Rec C _H - Human Isotype	Reference
B6B	Colorectal carcinoma	Y ¹	204
17-1A	Pancreatic carcinoma	Y ¹ , Y2 Y3 Y4	205, 205
17-1A	Pancreatic carcinoma	IgM	206
372.3	Pancreatic carcinoma	Y4 Y ¹	207, 208
372.3	Acute lymphocytic leukemia	Y ¹	209
NL-1	Human B cells	Y ¹	210
2H7	B cell lymphoma	Y ¹	
CAMPATH-1H	Human lymphocytes and monocytes	Y ¹	217
1G	Pancreatic carcinoma	Y ¹	211
CEM231 6-7	Colon carcinoma anti-CEA	Y ¹	212
CE25 B7	Anti-CEA	Y ¹	213
57-1G 10	Anti-CEA	Y ¹	214
MB1	Mammary carcinoma	Y ¹	215
Anti-TAC	T cells adult T cell leukemia	Y ¹	218

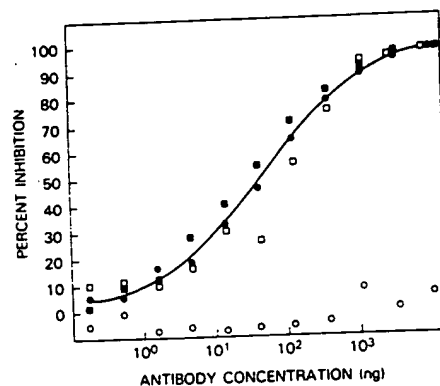


Figure 6.9. Comparative immunoreactivity of a native and recombinant chimeric mAb. Immunoreactivity of recombinant and recombinant:chimeric B72.3 were analyzed in a competitive radioimmunoassay and shown to be indistinguishable. Recombinant B72.3 (open squares) and recombinant:chimeric B72.3 (solid squares) were compared to the native B72.3 IgG standard (solid circles). MOPC-21 (open circles), an isotype-identical Ig, served as a negative control (216).

substantially less immunogenic than its murine counterpart.

The immune response to certain murine sequences in chimeric Igs could be reduced or eliminated by their substitution with human sequence, i.e., the Ig molecule could be "humanized." A complementary approach

to address this problem is to create a family of antibodies with the same combining specificities but with different idiotype determinants; these chimeric or humanized constructs could be used sequentially as a way of circumventing the human immune anti-idiotype response. Although the potential for the development of an anti-Id response following numerous administrations of recombinant/chimeric mAbs is real, it may not be as pronounced as that which has been seen when administering murine mAbs. The reason for this may be that the murine constant region is actually acting as a carrier and thus enhancing the immune response to the idiotype region. Thus, if the human Fc is less immunogenic in patients, a diminished anti-Id response may also be manifested.

One of the major advantages in the use of genetically engineered mAbs is the ability to modify the Ig molecule to alter pharmacokinetics. One may wish to slow down plasma clearance of a mAb, as in the case of using unconjugated antibody to mediate ADCC, or speed up the plasma clearance when one is using a mAb conjugated to a radionuclide, in which the circulating mAb-radionuclide may cause damage to marrow. Moreover, it is still unclear what the optimal Ig size and pharmacokinetics are for mAbs administered via intracavitary routes. Until now, immunochemists have been at the mercy of various enzymes such as papain to alter mAb size and

perhaps alter pharmacokinetics. However, these enzymes do not always work efficiently, and the resulting Ig fragments do not always demonstrate stability in vivo. Genetically engineered Igs provide several ways to alter pharmacokinetic properties. These include: (a) large alterations in size by the addition or deletion of domains and more subtle alterations in size by producing smaller deletions, (b) construction of Fv molecules, and (c) alterations in glycosylation. It has been demonstrated that minor changes in glycosylation can alter the serum pharmacokinetic patterns of macromolecules, and Igs in particular (219). Finally, alterations in Ig genes can be envisioned that will alter the metabolism or catabolism of the Ig. This is extremely important in the case of some mAb-isotope conjugates in which some isotopes may be retained in the liver or kidney. Size differences in Ig forms also have implications in tumor penetration. It has been shown that smaller mAb forms such as fragments will penetrate through a tumor mass more efficiently than intact Ig (150). Small, genetically engineered forms remain to be analyzed for this property.

Antitumor mAbs may also be modified so that they can act as more efficient vehicles for the delivery of antitumor drugs, toxins, radionuclides, or biological response modifiers. This can be achieved by directly ligating either the antitumor agents or their linkers into the Ig molecule. This has been accomplished in the ligation of *Pseudomonas* exotoxin into antitumor mAbs OVB3 and anti-TAC (42, 220, 221).

Finally, increases in affinity and avidity of antitumor mAbs have the obvious advantage of more efficient tumor binding and perhaps prolonged binding time at the tumor site. Genetically engineered mAbs can be modified to increase affinity in several ways. These include site-directed mutagenesis within the hypervariable region, altering glycosylation of the variable region, and perhaps altering the hinge region. Many of these manipulations have been carried out successfully with mAbs to nontumor antigens (222), and thus there is no apparent reason why they cannot ultimately be applied to antitumor clinical applications.

There is one point that should be emphasized as another major advantage in deal-

ing with genetically engineered molecules in cancer management. When a modification in the human Fc molecule has been made for one mAb, such as an alteration in size or glycosylation, or insertion of a drug or toxin, the cassette of this same construct can then be utilized with the antigen binding region of virtually any other anti-tumor mAb. Thus, the difficult and time consuming immunochemistry of linkage of an antitumor agent such as a drug, toxin, radionuclide, or biologic response modifier, or the enzymatic degradation of sugar moieties or Ig domains, does not have to be repeated for each antitumor mAb of interest. This property should thus greatly accelerate progress in the utilization of antitumor recombinant chimeric mAbs and their modified constructs in the various aspects of cancer diagnosis and therapy.

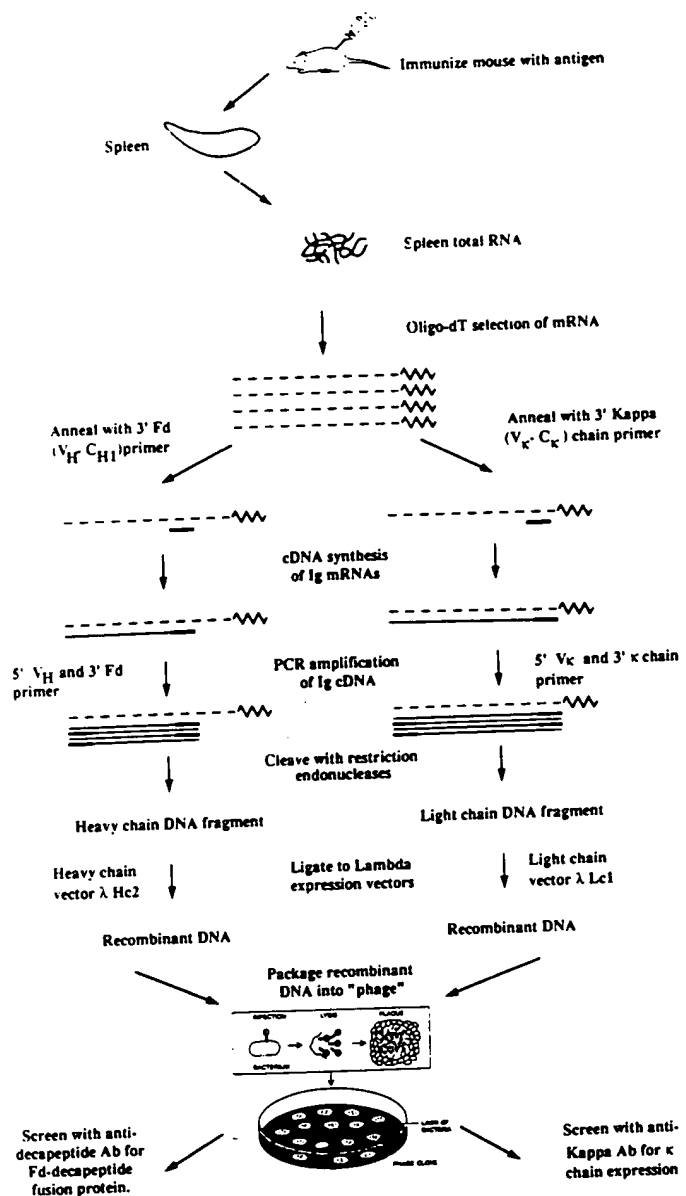
Single Chain Antigen Binding Proteins

One concern with the use of large molecules such as immunoglobulins as therapeutics is their inability to penetrate through large tumor masses (150); a smaller mAb form would be expected to exhibit better penetration. Furthermore, as detailed above, a major problem has been the appearance of the HAMA response in both diagnostic and therapeutic applications. Another problem that has emerged with the use of radiolabeled mAbs as therapeutics is that the radiolabeled mAb not bound to tumor remains in the circulation for several days with possible manifestation of marrow toxicity (129, 223); thus, a mAb form that clears the blood pool more rapidly would be advantageous. Whereas Flab[®]₂ and/or Fab' fragments help to reduce the HAMA response (109, 110), it is often difficult to generate these Ig forms in a manner such that they retain stable immunoreactivity in vivo.

One of the major recent advances in recombinant Ig technology has been the development of single chain antigen binding proteins, (SCAs) (119, 224, 225). These are recombinant proteins composed of an Ig variable light chain amino acid sequence (V_L) tethered to a variable heavy chain sequence (V_H) by a designed peptide that links the carboxyl terminus of the V_L to the amino terminus of the V_H or vice versa (see Fig. 6.10).

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Figure 6.10. Schematic diagram for the generation of a combinational library. See text and Reference 227 for details.

6. Monoclonal Antibodies: They're More and Less Than You Think 121

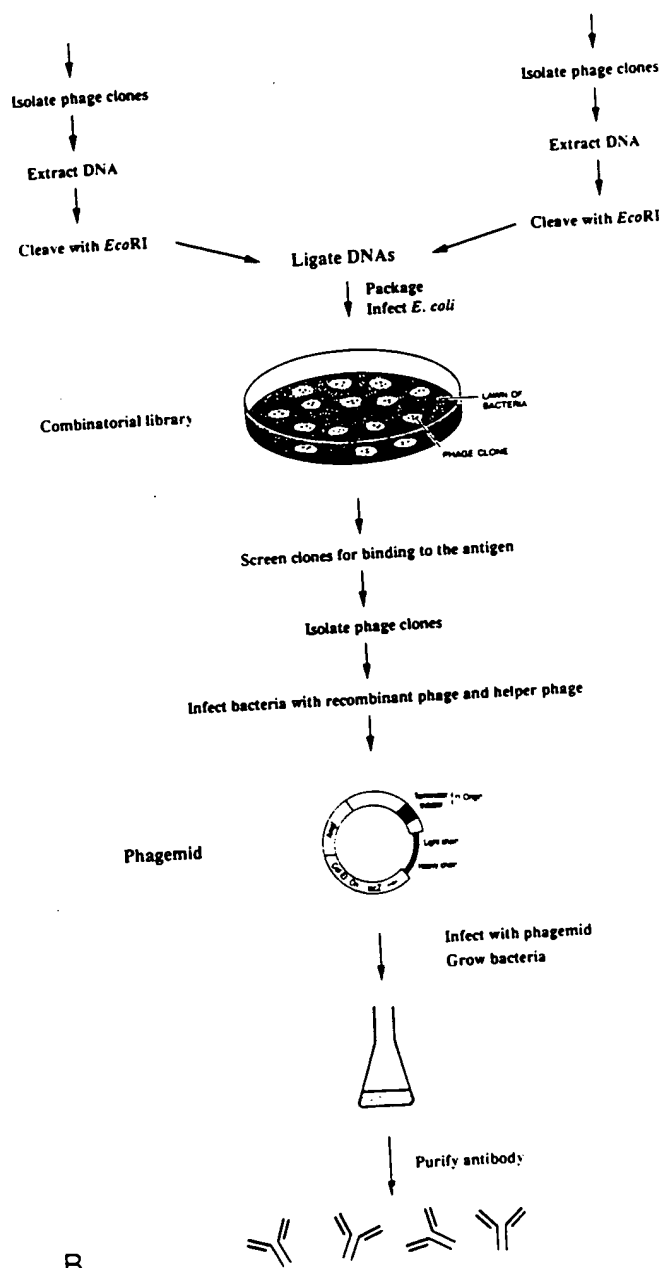


Figure 6.10. Constructing

A recent study (226) described the *in vivo* stability and application of an SCA. The molecule was a novel recombinant protein, expressed in *E. coli* and derived from the DNA sequence of the variable regions of the anti-tumor mAb B6.2. Competition RIA studies showed virtually the same binding properties for the SCA and Fab'. Comparative pharmacokinetic studies in athymic mice demonstrated the SCA to have much more rapid plasma clearance α and β phases ($t_{1/2}$ values of 2.4 minutes and 2.8 hours, respectively) than the Fab' fragment, as well as an extremely rapid whole body clearance. Despite its rapid clearance, the SCA showed a comparable uptake in a human tumor xenograft as that of the Fab' resulting in tumor:normal tissue ratios similar to or greater than those obtained with the Fab' (Table 6.6). Furthermore, the SCA molecule did not show accumulation in the kidney as did the Fab' or, as previously shown, Fab'₂ molecules. These studies thus demonstrated the apparent *in vivo* stability and the potential for clinical applications in cancer as well as other diseases for recombinant SCAs.

Table 6.6. Comparative red blood cell counts of single and multiple myeloma. SCA and a few cases of clearing myeloma on radiographs

Year	SO ₂		NO _x	
	1970	1974	1970	1974
Boston	0.04	0.06	3.37	5.26
New York	0.06	0.08	5.52	7.95
Scranton	0.11	0.10	4.08	3.10
Albany	0.14	0.38	3.10	3.55
London	0.03	0.45	3.90	3.80

[illegible]

nant:chimeric mAbs are now being used or being considered for use. These reasons include the following. (a) The very rapid clearance from the blood pool and whole body makes SCA molecules particularly attractive for coupling to radionuclides (and some drugs) so as to reduce unwanted bystander tissue toxicity. (b) The accumulation of Fab' and F(ab')₂ in the kidneys may not be seen with SCAs. This is potentially important for the radioimmunodetection of tumors in the peritoneal cavity and in reducing potential kidney toxicity associated with the use of drug- or radionuclide-conjugated Ig fragments. (c) The small size of SCAs should improve their capacity for rapid and evenly distributed penetration through tumor masses. (d) SCAs should have reduced immunogenicity because they do not contain C_H2 and C_H3 domains of intact Igs. of the C_H1 or C_L domains found in Fab' or F(ab')₂ fragments. The absence of C_H1 and C_L domains may also reduce antiallotype responses. (e) Because Fab' or F(ab')₂ fragments are generated through proteolysis, they are often difficult to produce in active form for *in vivo* use. In contrast, SCAs, which are synthesized from recombinant genes in *E. coli*, should be easier to make reproducibly in an immunoreactive form. (f) There should be a greatly reduced cost associated with the production, purification, and quality control of clinical grade

SCAs as compared to the use of conventional mAbs or mAb fragments. Moreover, one need not be concerned about contamination with mammalian DNA (i.e., oncogenes), mammalian retroviruses, and murine adventitious viruses (such as hepatitis, etc.) as in the case of conventional mAbs produced in mammalian cells. One also does not have the extra costly procedure of generating a proteolytic fragment from an intact IgG under the strict guidelines required for use as a clinical reagent. (g) Because SCA molecules are so small, it may be possible to add regions of the Ig molecule responsible for effector- or complement-mediated functions, or drugs or specific combining sites for drugs and radionuclides (i.e., bifunctional chelates) for more efficient therapeutic and/or diagnostic applications. This has recently been done with the anti-TAC molecule (225). Recent studies have thus demonstrated that SCA molecules can now be seriously considered for clinical applications.

COMBINATORIAL LIBRARIES

One of the more provocative recent findings has been the demonstration that one can produce recombinant Igs via a combinatorial library (227). A novel bacteriophage λ vector system was used to express in *E. coli* a combinatorial library of Fab fragments of the murine antibody repertoire. This system potentially allows for a rapid and easy identification of monoclonal Fab fragments in a form suitable for genetic manipulation. Via this approach, it was demonstrated possible to generate mAb Fab fragments against a transitional state analogue hapten. One possibility is that this method may complement and perhaps ultimately supersede the present day hybridoma technology. The basis for this approach is that current methods of generating mAbs may not provide for an adequate survey of the potential Ig gene repertoire.

Recent reports (215, 228) described the construction of a plasmid expression library in *E. coli* in which V_H fragments with reactivity to keyhole limpet hemocyanin (KLH) and lysozyme were isolated. Many believe, however, that it is unlikely that the affinity of isolated V_H fragments will generally match that of intact Igs, since the V_L has been shown to

be required for providing contacts critical for antigen binding (229).

The key to the combinatorial library process recently described (227) was in solving the problem of expressing the repertoires of heavy and light chains in combination. The combinatorial library was generated in two steps (Fig. 6.11). First, separate heavy and light chain libraries were constructed in two different λ phage vectors, each carrying a unique restriction endonuclease site placed antisymmetrically. The combinatorial construct was generated by cutting DNAs isolated from light and heavy chain libraries at the antisymmetric endonuclease site and rejoining the resulting phage arms.

To construct heavy and light chain libraries, mRNA was isolated from a mouse spleen that had been immunized with KLH-coupled *p*-nitrophenyl phosphoramidate (NPN) antigen. The 3' primers of heavy and light chain were used to synthesize cDNA copies of the V_H and V_L genes (Fig. 6.11). Amplification of the immunoglobulin genes was carried out by the polymerase chain reaction (PCR) technique using 3' primers and a battery of 5' primers specific for the heavy and the light chain genes. The amplified sequences were cloned in λ expression vectors genetically engineered for the expression of heavy and light chain gene sequences. The vectors included genetic elements essential for expression and secretion of the Fab fragments.

All the libraries (the light, the heavy, and Fab combinatorial) were screened to determine whether they contained recombinant phage that expressed V_H , V_L , or Fab fragments. Screening was performed by lifting phage plaques onto nitrocellulose filters and carrying out the filter binding assay, using the appropriate probes. Antibody to κ chain was used to screen plaques expressing light chain. To facilitate screening of the plaques expressing Fd (V_H - C_H1) sequences, a sequence encoding a decapeptide was "stitched" into the heavy chain expression vector. The availability of mAbs to this decapeptide made it convenient to screen Fd expressing plaques and to subsequently purify fusion protein by immunoaffinity. Thus, the combinatorial library was expressed in two steps. First, separate heavy and light chain

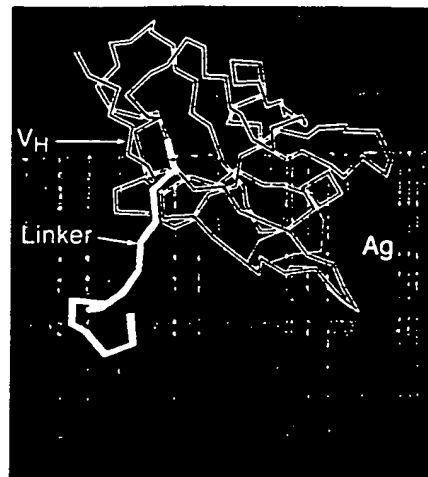


Figure 6.11. Schematic diagram of a single chain antigen binding protein (SCA). Note that the linker is distant to the binding region so as to not interfere with antigen binding. Red, V_H; blue, V_L; yellow, linker; white, antigen. See Reference 119 and text for details.

libraries were constructed in λ Hc2 and λ Lc1 vectors, respectively. Then, these two libraries were combined at the antisymmetric *EcoRI* sites present in each vector. This resulted in a library of clones, each of which potentially coexpress a heavy and light chain. These actual combinations are thus random and do not necessarily reflect the combinations present in B cells of the parent spleen. Phage clones coexpressing the light and heavy chain fragments (Fabs) were identified by binding to the NPN antigen coupled to 125 I-labeled bovine serum albumin (BSA). Screening of one million phage plaques from the combinatorial library identified approximately 100 clones that bound antigen (efficiency of 1 in 10,000). Resulting clones were shown to be producing the expected 50-kDa Fab protein.

While the studies reported (227) demonstrate the feasibility of this approach, only further studies will define its potential for widespread applications in the development of novel murine and human mAbs. As discussed, the size of the mammalian Ig repertoire is often approximated at 10^6 to 10^7 different antigen specificities. There is no reason to assume that a similar or larger phage combinatorial library cannot be constructed. One potential advantage of the phage

library is that factors such as antigen tolerance may restrict the expression of certain Ig specificities from the *in vivo* repertoire, but not the phage library. This may be extremely important in the area of antitumor antibodies where the host may be tolerant to certain TAAs. Moreover, obtaining Fab fragments from a combinatorial library does not involve the tissue culture procedures necessary for obtaining mAbs from the *in vivo* library. Studies are now ongoing to prepare a human combinatorial library. Finally, obtaining mAbs Fabs of murine or human origin from the phage combinatorial library gives one a *de facto* recombinant Ig fragment, which is ready for further genetic manipulation such as described earlier in this chapter.

It is thus anticipated that the merging of mAb immunochemistry and molecular biology will now bring us to an era of accelerated progress in mAb-based therapies.

REFERENCES

1. Göttinger HG, Funke I, Johnson JP, Gokel JM, Riethmüller G. The epithelial cell surface antigen 17-1A, a target for antibody-mediated tumor therapy: Its biochemical nature, Tissue distribution and recognition

- by different monoclonal antibodies. *Int J Cancer* 1986;38:47-53.
2. Herlyn D, Herlyn M, Ross AH, Ernst CS, Atkinson B, Koprowski H. Efficient selection of human tumor growth-inhibiting monoclonal antibodies. *J Immunol Meth* 1984;73:157-167.
3. Colcher D, Horan Hand P, Nuti M, Schlom J. A spectrum of monoclonal antibodies reactive with human mammary tumor cells. *Proc Natl Acad Sci USA* 1981;78:3199-3203.
4. Muraro R, Kuroki M, Wunderlich D, et al. Generation and characterization of B72.3 second generation monoclonal antibodies reactive with the tumor-associated glycoprotein 72 antigen. *Cancer Res* 1988;48:4588-4596.
5. Hellstrom I, Beaumier P, Hellstrom K. Antitumor effects of L6, an IgG2a antibody that reacts with most human carcinomas. *Proc Natl Acad Sci USA* 1986;83:7059-7063.
6. Tagliabue E, Menard S, Torre GD, et al. Generation of monoclonal antibodies reacting with human epithelial ovarian cancer. *Cancer Res* 1985;45:379-385.
7. Prat M, Griselli B, Bernardi A, et al. Expression of the monoclonal antibody-defined CAR-3 epitope on neoplastic and preneoplastic lesions of the colon mucosa. *Eur J Cancer Clin Oncol* 1987;23:923-932.
8. Arklie J, Taylor-Papadimitriou J, Bodmer W, Egan M, Millis R. Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. *Int J Cancer* 1981;28:23-29.
9. Krohn K, Helle M. Recognition with a monoclonal antibody of a cytoplasmic mammary carcinoma antigen, correlated to the estrogen receptor status. *Int J Cancer* 1986;37:43-47.
10. Kufe D, Inghirami G, Abe M, Hayes D, Just-Wheeler H, Schlom J. Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. *Hybridoma* 1994;3:223-232.
11. Hilken J, Hilgers J, Buijs F, et al. Monoclonal antibodies against human milk fat globule membrane useful in carcinoma research. In: Peeters H, ed. *Protides of the biological fluids*. United Kingdom: Pergamon Press, 1983:1013-1015.
12. Hilken J, Buijs F, Hilgers J, et al. Monoclonal antibodies against human milk fat globule membranes detecting differentiation antigens of the mammary gland and its tumors. *Int J Cancer* 1984;34:197-206.
13. Croghan GA, Papsidero LD, Valenzuela LA, Nemoto T, Penetrante R, Chu TM. Tissue distribution of an epithelial and tumor-associated antigen recognized by monoclonal antibody F36 22. *Cancer Res* 1983;43:4980-4988.
14. Thompson CH, Jones SL, Whitehead RII, McKenzie IF. A human breast tissue-associated antigen detected by a monoclonal antibody. *J Natl Cancer Inst* 1983;70:409-419.
15. Edwards D, Grzyb K, Dressler L, et al. Monoclonal antibody identification and characterization of a M_r 43,000 membrane glycoprotein associated with human breast cancer. *Cancer Res* 1986;46:1306-1317.
16. Menard S, Tagliabue E, Canevari S, Fossati G, Colnaghi MI. Generation of monoclonal antibodies reacting with normal and cancer cells of human breast. *Cancer Res* 1983;43:1295-1300.
17. Iacobelli S, Arno E, D'Orazio A, Coletti G. Detection of antigens recognized by a novel monoclonal antibody in tissue and serum from patients with breast cancer. *Cancer Res* 1986;46:3005-3010.
18. Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Soma Cell Genet* 1979;5:957-972.
19. Moriarty DM, Fox N, Aden DP, Hoyer JR, Knowles BB. Identification of human hepatoma defined cell surface molecules. *Hybridoma* 1983;2:39-47.
20. Watanabe M, Hirohashi S, Shimosato Y, et al. Carbohydrate antigen defined by a monoclonal antibody raised against a gastric cancer xenograft. *Jpn J Cancer Res* 1985;76:43-52.
21. Hollingsworth MA, Metzgar RS. Antigens of normal and malignant human exocrine pancreatic cells. In: Sell S, Reisfeld RA, eds. *Monoclonal antibodies in cancer*. Clifton, NJ: Humana Press, 1985:279.
22. Chin J, Miller F. Identification and localization of human pancreatic tumor-associated antigens by monoclonal antibodies to RWP-1 and RWP-2 cells. *Cancer Res* 1985;45:1723-1729.
23. Mach JP, Buchegger F, Forni M, et al. Use of a radiolabeled monoclonal anti-CEA antibodies for the detection of human carcinomas by external photoscanning and tomoscintigraphy. *Immunol Today* 1981;2:239-249.
24. Beatty JD, Duda RB, Williams LE, et al. Preoperative imaging of colorectal carcinoma with ¹¹¹In-labeled anticarcinoembryonic antigen monoclonal antibody. *Cancer Res* 1986;46:6494-6502.

25. Kuroki M, Kuroki M, Koga Y, Matsuoka Y. Monoclonal antibodies to carcinoembryonic antigen: A systematic analysis of antibody specificities by using related normal antigens and evidence for allotypic determinants on carcinoembryonic antigen. *J Immunol* 1984;133:2090-2097.
26. Primus FJ, Newell KD, Blue A, Goldenberg DM. Immunological heterogeneity of carcinoembryonic antigen: Antigenic determinants on carcinoembryonic antigen distinguished by monoclonal antibodies. *Cancer Res* 1983;43:686-692.
27. Muraro R, Wunderlich D, Thor A, et al. Definition by monoclonal antibodies of a repertoire on carcinoembryonic antigen differentially expressed in human colon carcinomas versus normal adult tissues. *Cancer Res* 1985;45:5769-5780.
28. Pimm MV, Perkins AC, Baldwin RW. Simultaneous localization of two monoclonal antibodies in a human colon carcinoma xenograft. *IRCS Med Sci* 1985;13:499-500.
29. Vecchi M, Sakamaki S, Diamond B, Novikoff A, Das K. Development of a monoclonal antibody reactive to gastrointestinal goblet cells. *Proc Natl Acad Sci USA* 1987;84:3425-3429.
30. Gottlinger H, Lobo F, Grimm T, Riethmüller G, Johnson JP. Biochemical characterization and tissue distribution of the cora antigen, a cell surface glycoprotein differentially expressed on malignant and benign gastrointestinal epithelia. *Cancer Res* 1988;48:2198-2203.
31. Muraro R, Nuti M, Natali PG, et al. A monoclonal antibody (D612) with selective reactivity for malignant and normal gastrointestinal epithelium. *Int J Cancer* 1989;43:598-607.
32. Muraro R, Wunderlich D, Thor A, Cunningham R, Noguchi P, Schlom J. Immunological characterization of a novel human colon associated antigen (CAA) by a monoclonal antibody. *Int J Cancer* 1987;39:34-44.
33. Papsidero L, Croghan G, Wang M, et al. Monoclonal antibody (F5) to human prostate antigen. *Hybridoma* 1983;2:139-147.
34. Frankel A, Rouse R, Wang M, Chu T, Herzenberg L. Monoclonal antibodies to a human prostate antigen. *Cancer Res* 1982;42:3714-3718.
35. Chu TM, Murphy GP. What's new in tumor markers for prostatic cancer? *Urology* 1986;27:487-491.
36. Wright G, Beckett M, Starling J, et al. Immunohistochemical localization of prostate carcinoma-associated antigens. *Cancer Res* 1983;43:5509-5516.
37. Ware JL, Paulson DF, Parks SF, Webb KSO. Production of monoclonal antibody α Pro3 recognizing a human prostatic carcinoma antigen. *Cancer Res* 1982;42:1215-1222.
38. Starling J, Sieg S, Beckett M, Schellhammer P, Ladaga L, Wright G Jr. Monoclonal antibodies to human prostate and bladder tumor-associated antigens. *Cancer Res* 1982;42:3084-3089.
39. Fradet Y, Cordon-Cardo C, Whitmore WF, McLamed M, Old L. Cell surface antigens of human bladder tumors: definition of tumor subsets by monoclonal antibodies and correlation with growth characteristics. *Cancer Res* 1986;46:5183-5188.
40. Masuko T, Yagita H, Hashimoto Y. Monoclonal antibodies against cell surface antigens present on human urinary bladder cancer cells. *J Natl Cancer Inst* 1984;72:523-530.
41. Young D, Prout G, Lin C. Production and characterization of mouse monoclonal antibodies to human bladder tumor-associated antigens. *Cancer Res* 1985;45:4439-4446.
42. Willingham MC, FitzGerald DJ, Pastan I. *Pseudomonas* exotoxin coupled to a monoclonal antibody against ovarian cancer inhibits the growth of human ovarian cancer cells in a mouse model. *Proc Natl Acad Sci USA* 1987;84:2474-2478.
43. Bast RC Jr, Freeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. *J Clin Invest* 1981;68:1331-1337.
44. De Kreiser T, Thorne H, Jacobs D, Jose D. The sebaceous gland antigen defined by the OM-1 monoclonal antibody is expressed at high density on the surface of ovarian carcinoma cells. *Eur J Cancer Clin Oncol* 1985;21:1019-1035.
45. Knauf S, Kalwas J, Helmkamp BF. Monoclonal antibodies against human ovarian tumor associated antigen NB/70K: Preparation and use in a radioimmunoassay for measuring NB/70K in serum. *Cancer Immunol Immunother* 1986;21:217-225.
46. Miott S, Canevari S, Menard D, et al. Characterization of human ovarian carcinoma-associated antigens defined by novel monoclonal antibodies with tumor-restricted specificity. *Int J Cancer* 1987;39:297-303.
47. Berkowitz RS, Kabawat S, Lazarus H, Colvin R, Knapp R, Bast R Jr. Comparison of a rabbit heteroantiserum and a murine mon-

- monoclonal antibody raised against a human epithelial ovarian carcinoma cell line. *Am J Obstet Gynecol* 1983;146:607-612.
48. Battacharya M, Chatterjee SK, Bartlow JJ. Monoclonal antibodies recognizing tumor associated antigen of human ovarian mucinous cystadenocarcinomas. *Cancer Res* 1982;42:1650-1654.
49. Poels LG, Peters D, van Megen Y, et al. Monoclonal antibody against human ovarian tumor associated antigens. *J Natl Cancer Inst* 1986;76:781-791.
50. Bunn PA Jr, Linnoila I, Minna JD, Carney D, Gazdar AF. Small cell lung cancer, endocrine cells of the fetal bronchus, and other neuroendocrine cells express the Leu-7 antigenic determinant present on natural killer cells. *Blood* 1985;65:764-768.
51. Watanabe J, Okabe T, Fujisawa M, Takaku F, Hirohashi S, Shimamoto Y. Monoclonal antibody that distinguishes small-cell lung cancer from non-small-cell lung cancer. *Cancer Res* 1987;47:826-829.
52. Varki NM, Reisfeld RA, Walker LE. Antigens associated with human lung adenocarcinoma defined by monoclonal antibodies. *Cancer Res* 1984;44:681-687.
53. Postmus P, Hirschler-Schulte T, Leij L, et al. Diagnostic application of a monoclonal antibody against small cell lung cancer. *Cancer* 1986;57:60-63.
54. Kyoizumi S, Akiyama M, Kouno N, et al. Monoclonal antibodies to human squamous cell carcinoma of the lung and their application to tumor diagnosis. *Cancer Res* 1985;46:3274-3281.
55. Reeve JG, Wulfrank DA, Stewart J, Twentymann PR, Baillie-Johnson H, Bleehen NM. Monoclonal-antibody-defined lung tumor cell-surface antigens. *Int J Cancer* 1985;35:769-775.
56. Pettijohn D, Stranahan P, Due C, Ronne E, Sorensen HR, Olsson L. Glycoproteins distinguishing non-small cell from small cell human lung carcinoma recognized by monoclonal antibody 43-9F. *Cancer Res* 1987;47:1161-1169.
57. Dazord L, Bourel D, Martin A, et al. A monoclonal antibody (Po6E) directed against human lung squamous cell carcinoma. Immunolocalization of tumor xenografts in nude mice. *Cancer Immunol Immunother* 1987;24:263-268.
58. Hellstrom I, Garrigues HJ, Cabasco L, Mosely GH, Brown JP, Hellstrom KE. Studies of a high molecular weight human melanoma-associated antigen. *J Immunol* 1983;130:1467-1472.
59. Goodman GE, Beaumier P, Hellstrom I, Fernyhough B, Hellstrom KE. Pilot trial of murine monoclonal antibodies in patients with advanced melanoma. *J Clin Oncol* 1985;3:340-352.
60. Houghton AN, Mintzer D, Cordon-Cardo C, et al. Mouse monoclonal IgG3 antibody detecting G₁₅ ganglioside: A phase I trial in patients with malignant melanoma. *Proc Natl Acad Sci USA* 1985;82:1242-1246.
61. Honsik CJ, Jung C, Reisfeld RA. Lymphokine-activated killer cells targeted by monoclonal antibodies to the disialogangliosides GD2 and GD3 specifically lyse human tumor cells of neuroectodermal origin. *Proc Natl Acad Sci USA* 1986;83:7893-7897.
62. Liao S-K, Clarke BJ, Khosravi M, Kwong PC, Brickenden A, Dent PB. Human melanoma-specific oncofetal antigen defined by mouse monoclonal antibody. *Int J Cancer* 1982;30:573-580.
63. Morgan AC Jr, Galloway DR, Reisfeld RA. Production and characterization of monoclonal antibody to a melanoma specific glycoprotein. *Hybridoma* 1981;1:27-36.
64. Salk D, et al. Technetium-labeled monoclonal antibodies for imaging metastatic melanoma: Results of a multicenter clinical study. *Semin Oncol* 1988;15:608-618.
65. Schnegg JF, Diserens AC, Carrel S, Accolla RS, deTribolet N. Human glioma-associated antigens detected by monoclonal antibodies. *Cancer Res* 1981;41:1209-1213.
66. Jennings MT, Jennings VDL, Asadourian LH, et al. Five novel cell surface antigens of CNS neoplasms. *J Neurol Sci* 1989;89:63-78.
67. Nai-Kong VC, Lazarus H, Miraldi FD, et al. Ganglioside G₁₅ specific monoclonal antibody 3F5: A phase I study in patients with neuroblastoma and malignant melanoma. *J Clin Oncol* 1987;5:1430-1440.
68. Waldmann TA, Kozak RW, Tsudo M, Ohishi T, Bongiovanni KF, Goldman CK. Receptors in adult T-cell leukemia: A target for immunotherapy. In: Neth R, Gallo RC, Greaves MF, Kabisch H, eds. *Hematology and blood transfusion*. Vol. XXXI. Heidelberg: Springer-Verlag, 1987:110-115.
69. Dillman RO, Beauregard J, Shawler DL, et al. Continuous infusion of T101 monoclonal antibody in chronic lymphocytic leukemia and cutaneous T-cell lymphoma. *J Biol Res Med* 1986;5:394-410.
70. Miller RA, Maloney DG, McKillop J, Levy R. In vivo effects of murine hybridoma monoclonal antibody in a patient with T-cell leukemia. *Blood* 1981;58:78-86.

71. Reinherz EL, Schlossman SF. The differentiation and function of human T lymphocytes: A review. *Cell* 1980;19:821-827.
72. Freedman A, Nadler L. Cell surface markers in hematologic malignancies. *Semin Oncol* 1987;14:193-212.
73. Nadler L. B cell leukemia panel workshop. In: Reinherz EL, Haynes BF, Nadler LM, Bernstein ID, eds. *Leukocyte typing II*. Vol. 2. New York: Springer-Verlag, 1986:3-43.
74. Griffin JD, Linch D, Sabbath KD, Larcom P, Schlossman SF. A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. *Leuk Res* 1987;8:521-534.
75. Miller RA, Maloney DG, Warnke R, Levy R. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *N Engl J Med* 1982;306:517-522.
76. DeNardo SJ, DeNardo GL, O'Grady LF, et al. Treatment of B cell malignancies with 131I Lym-1 monoclonal antibodies. *Int J Cancer Suppl* 1988;3:96-101.
77. Henslee PJ, Byers VS, Jennings CD, et al. A new approach to the prevention of graft-versus-host disease using XomaZyme-H65 following histo-incompatible partially T-depleted marrow grafts. *Transplant Proc* 1989;21:3004-3007.
78. Press OW, Martin PJ, Thorpe PE, Vitetta ES. Ricin A-chain containing immunotoxins directed against different epitopes on the CD2 molecule differ in their ability to kill normal and malignant T cells. *J Immunol* 1988;141:4410-4417.
79. Press OW, Eary JF, Badger CC, et al. Treatment of refractory non-Hodgkin's lymphoma with radiolabeled MB-1 (anti-CD37) antibody. *J Clin Oncol* 1989;7:1027-1038.
80. Symonds EM, Perkins AC, Pimm MV, Baldwin RW, Hardy JG, Williams DA. Clinical implications for immuno-scintigraphy in patients with ovarian malignancy: A preliminary study using monoclonal antibody 791T-36. *J Obstet Gynecol* 1985;92:270-276.
81. Schlom J, Wunderlich D, Teramoto YA. Generation of human monoclonal antibodies reactive with human mammary carcinoma cell antigens. *Proc Natl Acad Sci USA* 1980;77:6841-6845.
82. Haspel MV, McCabe RP, Pomato N, Hoover HC, Hanna MG. Human monoclonal antibodies: Generation of tumor cell reactive monoclonal antibodies using peripheral blood lymphocytes from actively immunized patients with colorectal carcinoma. In: Reisfeld R, Sell S, eds. *Monoclonal antibodies and cancer therapy*. New York: Alan R Liss, 1985:505.
83. Cote RJ, Morrissey DM, Houghton AN, Beatrice EJ, Oetting HF, Old LJ. Generation of human monoclonal antibodies reactive with cellular antigens. *Proc Natl Acad Sci USA* 1983;80:2026-2030.
84. Sugiyama T, Imai K, Masukawa J, et al. Preparation of human monoclonal antibodies of IgG type to gastrointestinal cancer associated antigen. *Jpn J Cancer Res* 1986;77:722-729.
85. Irie RF, Morton DL. Regression of cutaneous metastatic melanoma by intrasional injection with human monoclonal antibody to ganglioside GD2. *Proc Natl Acad Sci USA* 1986;83:8694-8698.
86. Imam A, Drushella MM, Taylor CR, Tokes ZA. Generation and immunohistological characterization of human monoclonal antibodies to mammary carcinoma cells. *Cancer Res* 1985;45:263-271.
87. Lloyd KO, Old LJ. Human monoclonal antibodies to glycolipids and other carbohydrate antigens: Dissection of the humoral immune response in cancer patients. *Cancer Res* 1989;49:3445-3451.
88. Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res* 1984;44:1002-1007.
89. Drebin JA, Link VC, Weinberg RA, Greene MI. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. *Proc Natl Acad Sci USA* 1986;83:9129-9133.
90. Raychaudhuri A, Saeki Y, Fujii H, Kohler H. Tumor specific idiotype vaccines. I. Generation and characterization of internal image tumor antigens. *J Immunol* 1986;137:1743-1749.
91. Nepom GT, Nelson KA, Holbeck SL, Hellstrom I, Hellstrom KE. Induction of immunity to a human tumor marker by in vivo administration of anti-idiotypic antibodies in mice. *Proc Natl Acad Sci USA* 1984;81:2864-2867.
92. Herlyn D, Wettendorff M, Schmoll E, et al. Anti-idiotypic immunization of cancer patients: Modulation of the immune response. *Proc Natl Acad Sci USA* 1987;84:8055-8059.
93. Perosa F, Ferrone S. Syngeneic antidiotypic monoclonal antibodies to the murine anti-HLA-DR. DP monoclonal antibody CR11-462. *Hum Immunol* 1988;23:255-269.
94. Cote RJ. Analysis of the human immune repertoire using human monoclonal antibodies. *Intl J Biol Markers* 1989;4:59-64.
95. Schlom J, Weeks MO. Potential clinical util-

- ity of monoclonal antibodies in the management of human carcinomas. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Important advances in oncology*. Philadelphia: JB Lippincott, 1985:170-192.
96. Boyer CM, Lidor Y, Lottich SC, Bast RC Jr. Antigenic cell surface markers in human solid tumors. *Antibody Immunocnj Radiopharmacol* 1988;1:105-162.
 97. Freedman AS, Nalder LM. Cell surface markers in hematologic malignancies. *Semin Oncol* 1987;14:193-212.
 98. Eckhardt, Holzner, Nagel, eds. *Contributions to oncology*. Vol. 32. Munich, Germany: JB Lippincott, 1988.
 99. Kuroki M, Greiner J, Simpson J, Primus F, Guadagni F, Schlom J. Serologic mapping and biochemical characterization of the carcinoembryonic antigen epitopes using distinct monoclonal antibodies. *Int J Cancer* 1989;44:208-218.
 100. Hammarstrom S, Shively JE, Paxton RJ, et al. Antigen sites in carcinoembryonic antigen. *Cancer Res* 1989;49:4852-4858.
 101. Shuke N, Steis RG, McCabe RP, et al. Pharmacokinetics of two human IgM monoclonal antibodies (16.88 and 28A32) [Abstract]. *J Nucl Med* 1989;30:909.
 102. Soper JT, Hunter VJ, Daly L, Tanner M, Creasman WT, Bast RC Jr. Preoperative serum tumor-associated antigen levels in women with pelvic masses. *Obstet Gynecol* 1990;75:249-254.
 103. Ohuchi N, Takahashi K, Matoba N, et al. Comparison of serum assays for TAG-72, CA19-9 and CEA in gastrointestinal carcinoma patients. *Jpn J Clin Oncol* 1989;19:242-248.
 104. Colcher D, Milenic DE, Ferroni P, et al. In vivo fate of monoclonal antibody B72.3 in patients with colorectal cancer. *J Nucl Med* 1990;31:1133-1142.
 105. Steplewski Z, Sun LK, Shearman CW, Ghayeb J, Daddona P, Koprowski H. Biological activity of human-mouse IgG1, IgG2, IgG3, and IgG4 chimeric monoclonal antibodies with antitumor specificity. *Proc Natl Acad Sci USA* 1988;85:4852-4856.
 106. Munn DH, Cheung NKV. Interleukin-2 enhancement of monoclonal antibody-mediated cellular cytotoxicity against human melanoma. *Cancer Res* 1987;47:6600-6605.
 107. Mujoo K, Cheresch DA, Yang HM, Reitsfeld RA. Distal ganglioside G_{D2} on human neuroblastoma cells: Target antigen for monoclonal antibody-mediated cytotoxicity and suppression of tumor growth. *Cancer Res* 1987;47:1098-1104.
 108. Grob JP, Mach JP, Buchegger F, et al. Cancer detection by immunoscintigraphy: selection, and clinical application of monoclonal anti-CEA antibodies and their Fab' ₂ or Fab fragments. *Cancer Detect Immunoscintigraphy* 1983;73-79.
 109. Reynolds JC, DeVecchio S, Sakahara H, et al. Anti-murine response to mouse monoclonal antibodies: Clinical findings and implications. *Nucl Med Biol* 1989;16:121-125.
 110. Seccamani E, Tattaneli M, Mariani M, Spranzi E, Scassellati GA, Siccardi AG. A simple qualitative determination of human antibodies to murine immunoglobulins (HAMA) in serum samples. *Nucl Med Biol* 1989;16:167-170.
 111. Levy R, Miller RA. Tumor therapy with monoclonal antibodies. *Fed Proc* 1983;42:2650-2656.
 112. LoBuglio AF, Wheeler RH, Trang J, et al. Mouse/human chimeric monoclonal antibody in man: Kinetics and immune response. *Proc Natl Acad Sci USA* 1989;86:4220-4224.
 113. Sears HF, Herlyn D, Steplewski Z, Koprowski H. Effects of monoclonal antibody immunotherapy on patients with gastrointestinal adenocarcinoma. *J Biol Res Mod* 1984;3:138-150.
 114. Colcher D, Esteban JM, Carrasquillo JA, et al. Quantitative analyses of selective radiolabeled monoclonal antibody localization in metastatic lesions of colorectal cancer patients. *Cancer Res* 1987;47:1185-1189.
 115. Schneck DW, Petersen B, Zimmerman J, Butler F, Dugan W. Phase I studies with a monoclonal antibody vinca conjugate (MC, KS1/4-DAVLB) in patients with adenocarcinoma [Abstract]. *The Fourth International Conference on Monoclonal Antibody Immunocjugates for Cancer*. San Diego, CA, 1989.
 116. Weiner LM, O'Dwyer J, Kitson J, et al. Phase I evaluation of an anti-breast carcinoma monoclonal antibody 260F9-recombinant ricin A chain immunocjugate. *Cancer Res* 1989;49:4062-4067.
 117. Reichmann L, Clark MR, Waldmann H, Winter G. Reshaping human antibodies for therapy. *Nature* 1988;332:323-327.
 118. Queen C, Schneider WP, Selick HE, et al. A humanized antibody that binds to the interleukin 2 receptor. *Proc Natl Acad Sci USA* 1989;86:10029-10033.
 119. Bird RE, Hardman KD, Jacobson JW, et al. Single-chain antigen-binding proteins. *Science* 1988;242:423-426.
 120. Jain RK. Transport of molecules in the tumor interstitium: A review. *Cancer Res* 1987;47:3039-3051.

121. Schlom J, Molinolo A, Simpson JF, et al. Advantage of dose fractionation in monoclonal antibody-targeted radioimmunotherapy. *J Natl Cancer Inst* 1990; 82:763-771.
122. Starling JJ, Hinson NA, Marder P, Maciak RS, Laguzza BC. Rapid internalization of antigen-immunoconjugate complexes is not required for anti-tumor activity of monoclonal antibody-drug conjugates. *Antibody Immunoconj Radiopharm* 1988;1:311-324.
123. Brechbiel MW, Gansow OA, Atcher RW, et al. Synthesis of 1-(p-isothiocyanatobenzyl) derivatives of DTPA and EDTA. Antibody labeling and tumor imaging studies. *Inorganic Chem* 1986;25:2772-2781.
124. Esteban JM, Colcher D, Sugarbaker P, et al. Quantitative and qualitative aspects of radiolocalization in colon cancer patients of intravenously administered mAb 72.3. *Int J Cancer* 1987;39:50-59.
125. Mach JP, Chatal JF, Lumbroso JD, et al. Tumor localization in patients by radiolabeled monoclonal antibodies against colon carcinoma. *Cancer Res* 1983;43:5593-5600.
126. Goldenberg DM, Kimm EE, Bennett SJ, Nelson MO, DeLand FH. Carcinoembryonic antigen radioimmunodetection in the evaluation of colorectal cancer in the detection of occult neoplasms. *Gastroenterology* 1983;84:524-532.
127. Stillwagon GB, Order SE, Klein JL, et al. Multi-modality treatment of primary non-resectable intrahepatic cholangiocarcinoma with ¹³¹I anti-CEA—A radiation therapy oncology group study. *J Rad Oncol Bio Phys* 1987;13:687-695.
128. Krishnamurthy S, Morris JF, Antonovic R, et al. Evaluation of primary lung cancer with indium 111 anti-carcinoembryonic antigen (type ZCE-025) monoclonal antibody scintigraphy. *Cancer* 1990;65:458-465.
129. Colcher D, Esteban J, Carrasquillo JA, et al. Complementation of intracavitary and intravenous administration of a monoclonal antibody (B72.3) in patients with carcinoma. *Cancer Res* 1987;47:4218-4224.
130. Lastoria S, D'Amico P, Mansi L, et al. A prospective imaging study of ¹³¹I-B72.3 monoclonal antibody in patients with epithelial ovarian cancer: Preliminary report. *Nuclear Medicine Communications* 1988;9:347-356.
131. Renda A, Salvatore M, Sava M, et al. Immunoscintigraphy in the follow-up of patients operated on for carcinoma of the sigmoid and rectum. *Dis Colon Rectum* 1987;30:683-686.
132. Raubitschek A, Colcher D, Lastoria S, et al. Intraperitoneally administered radiolabeled monoclonal antibodies in patients with peritoneal carcinomatosis [Abstract]. Second Conference on Radioimmunodetection and Radioimmunotherapy of Cancer. Princeton, NJ, 1988.
133. Moldofsky PJ, Powe J, Mulhern CB, et al. Metastatic colon carcinoma detected with radiolabeled F(ab')₂ monoclonal antibody fragments. *Radiology* 1983;149:549-555.
134. Pectasides D, Stewart S, Courtenay-Luck N, et al. Antibody-guided irradiation of malignant pleural and pericardial effusions. *Br J Cancer* 1986;53:727-732.
135. Larson SM, Carrasquillo JA, Krohn KA, et al. Localization of ¹³¹I-labeled p97-specific Fab fragments in human melanoma as a basis for radiotherapy. *J Clin Invest* 1983;72:2101-2114.
136. Coakham HB, Richardson RB, Davies AG, Bourne SP, Eckert H, Kemshead JT. Neoplastic meningitis from a pineal tumor treated by antibody-guided irradiation via the intrathecal route. *Br J Neurosurg* 1988;2:193-204.
137. Courtenay-Luck N, Epenetos AA, Halnan KE, et al. Antibody-guided irradiation of malignant lesions: Three cases illustrating a new method of treatment. *Lancet* 1441-1443, June 30, 1984.
138. Epenetos AA, Courtenay-Luck N, Pickering D, et al. Antibody guided irradiation of brain glioma by arterial infusion of radioactive monoclonal antibody against epidermal growth factor receptor and blood group A antigen. *Br Med J* 1985;290:1463-1466.
139. Lashford LS, Davies AG, Richardson RB, et al. A pilot study of ¹³¹I monoclonal antibodies in the therapy of leptomeningeal tumors. *Cancer* 1988;61:857-868.
140. Order SE, Klein JL, Lechner PK, Frincke J, Lollo C, Carlo DJ. ⁹⁰Yttrium antiferritin—A new therapeutic radiolabeled antibody. *J Rad Oncol Bio Phys* 1985;12:277-281.
141. Ward B, Mather S, Shepherd J, et al. The treatment of intraperitoneal malignant disease with monoclonal antibody guided ¹³¹I radiotherapy. *Br J Cancer* 1988;58:658-662.
142. Siccardi AG, Buraggi GL, Callegaro L, et al. Immunoscintigraphy of adenocarcinomas by means of radiolabeled F(ab')₂ fragments of an anti-carcinoembryonic antigen monoclonal antibody: A multicenter study. *Cancer Res* 1989;49:3095-3103.
143. Maguire TR, Schmelter RF, Pascucci VL, Conklin JJ. Immunoscintigraphy of colorectal adenocarcinoma: Results with site-specifically radiolabeled B72.3 (¹¹¹In-CYT-

1031. Antibody Immunocj Radiopharm 1989;2:257-269.
144. Cheung NK, Lazarus H, Miraldi FD, et al. Ganglioside G_{D2} specific monoclonal antibody 3F8: A phase I study in patients with neuroblastoma and malignant melanoma. *J Clin Oncol* 1987;5:1430-1440.
145. Order SE. Antibody Immunocj Radiopharm 1990;3:38-87.
146. Thor A, Ohuchi N, Szpak C, Johnston W, Schlom J. The distribution of oncofetal antigen TAG-72 defined by monoclonal antibody B72.3. *Cancer Res* 1986;46:3118-3124.
147. Thor A, Viglione MJ, Muraro R, Ohuchi N, Schlom J, Gorstein F. Monoclonal antibody B72.3 reactivity with human endometrium: A study of normal and malignant tissues. *Int J Gynecol Pathol* 1987;6:235-247.
148. Wolf BC, D'Emilia JC, Salem RR, et al. Detection of the tumor associated glycoprotein antigen (TAG-72) in premalignant lesions of the colon. *J Natl Cancer Inst* 1989;81:1913-1917.
149. Xu M, Real FX, Welt S, Schussler MH, Oettgen HF, Old LJ. Expression of TAG-72 in normal colon, transitional mucosa and colon cancer. *Int J Cancer* 1989;44:985-989.
150. Esteban JM, Schlom J, Mornex F, Colcher D. Radioimmunotherapy of athymic mice bearing human colon carcinomas with monoclonal antibody B72.3: Histologic and autoradiographic study of effects on tumors and normal organs. *Eur J Cancer* 1987;23:643-655.
151. Martin EW, Mojzsisik CM, Hinkle GH, et al. Radioimmunoguided surgery: A new approach to the intraoperative detection of tumor using monoclonal antibody B72.3. *Am J Surg* 1988;156:386-392.
152. Nieroda CA, Mojzsisik C, Sardi A, et al. Staging of carcinoma of the breast using a hand-held gamma detecting probe and monoclonal antibody B72.3. *Surg Gynecol Obstet* 1989;169:35-40.
153. Nadler LM, Stashenko P, Hardy R, et al. Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. *Cancer Res* 1980;40:3147-3154.
154. Ritz J, Pesando JM, Sallan SE, et al. Serotherapy of acute lymphoblastic leukemia with monoclonal antibody. *Blood* 1981;58:141-152.
155. Waldmann TA. Multichain interleukin-2 receptor: A target for immunotherapy in lymphoma. *J Natl Cancer Inst* 1989;81:914-923.
156. Waldmann TA, Goldman CK, Bongiovanni KF, et al. Therapy of patients with human T-cell lymphotropic virus I-induced adult T-cell leukemia with anti-Tac, a monoclonal antibody to the receptor for interleukin-2. *Blood* 1988;72:1805-1816.
157. Sears HF, Herlyn D, Steplewski A, Koprowski H. Phase II clinical trial of a murine monoclonal antibody cytotoxic for gastrointestinal adenocarcinoma. *Cancer Res* 1985;45:5910-5913.
158. LoBuglio AF, Saleh MN, Lee J, et al. Phase I trial of multiple large doses of murine monoclonal antibody CO17-1A. I. Clinical aspects. *J Natl Cancer Inst* 1988;80:932-936.
159. Foon KA, Schroff RW, Bunn PA, et al. Effects of monoclonal antibody therapy in patients with chronic lymphocytic leukemia. *Blood* 1984;64:1085-1093.
160. Bernstein N, Levy R. Treatment of a murine B cell lymphoma with monoclonal antibodies and IL-2. *J Immunol* 1987;139:971-976.
161. Kahn M, Hellstrom I, Estlin CD, Hellstrom KE. Monoclonal antidiotypic antibodies related to the p97 human melanoma antigen. *Cancer Res* 1989;49:3157-3162.
162. Fialkow PJ, Klein E, Klein G, Singh S. Immunoglobulin and glucose-6-phosphate dehydrogenase as markers of cellular origin in Burkitt's lymphoma. *J Exp Med* 1973;138:89-102.
163. Levy R, Warnke R, Dorfman RF, Hainovich J. The monoclonality of human B-cell lymphomas. *J Exp Med* 1977;145:1014-1028.
164. Salmon SE, Seligmann M. B cell neoplasia in man. *Lancet* 1974;ii:1230-1233.
165. Schroer KR, Briles DE, VanBoxel JA, Davie JM. Idiotype uniformity of cell surface immunoglobulin in chronic lymphocytic leukemia. Evidence for proliferation. *J Exp Med* 1974;146:1416-1420.
166. Levy R, Miller RA. Biological and clinical implications of lymphocyte hybridomas: Tumor therapy with monoclonal antibodies. *Annu Rev Med* 1983;34:107-116.
167. Hamblin TJ, Abdul-Ahad AK, Gordon J, Stevenson FK, Stevenson GT. Preliminary experience in treating lymphocyte leukemia with antibody to immunoglobulin idiotypes on the cell surface. *Br J Cancer* 1980;42:495-502.
168. Meeker TC, Lowder J, Maloney DG, et al. A clinical trial of anti-Id therapy for B-cell malignancy. *Blood* 1985;65:1349-1363.
169. Starnes CO, Carroll WL, Campbell MK, Houston LL, Apell G, Levy R. Heterogeneity of a murine B cell lymphoma: Isolation and characterization of idiotypic variants. *J Immunol* 1988;141:333-339.

170. Carroll WL, Starnes CO, Levy R, Levy S. Alternative V_H gene rearrangements in a murine B cell lymphoma: An explanation for idiotype heterogeneity. *J Exp Med* 1988;168:1607-1620.
171. Berinstein N, Campbell MJ, Lam K, Carswell C, Levy S, Levy R. Idiotype variation in a human B lymphoma cell line. *J Immunol* 1990;144:752-758.
172. Thielemans K, Maloney DG, Meeker T, et al. Strategies for production of monoclonal anti-idiotype antibodies against a human B cell lymphoma. *J Immunol* 1984;133:495-501.
173. Miller RA, Hart S, Samoszuk M, et al. Shared idiotypes expressed by human B-cell lymphomas. *N Engl J Med* 1989;321:851-857.
174. Brown SL, Miller RA, Horning SJ, et al. Treatment of B cell lymphomas with anti-idiotype antibodies alone and in combination with α IFN. *Blood* 1989;73:651-661.
175. Koprowski H, Herlyn D, Lubeck M, De-Freitas E, Sears HF. Human anti-idiotypic antibodies in cancer patients. Is the modulation of the immune response beneficial to the patient? *Proc Natl Acad Sci USA* 1984;81:216-219.
176. Wettendorf M, Iliopoulos D, Tempero M, et al. Idiotype cascades in cancer patients treated with monoclonal antibody CO17-1A. *Proc Natl Acad Sci USA* 1989;86:3787-3791.
177. Traub UC, DeJager RL, Primus FJ, Losman M, Goldenberg DM. Antidiotype antibodies in cancer patients receiving monoclonal antibody to carcinoembryonic antigen. *Cancer Res* 1988;48:4002-4006.
178. Ferrone S, Chen G, Yang H, Yamada M, Zheng Y, Mittelman A. Murine antidiotypic monoclonal antibodies in melanoma. Immunochemical characterization and clinical applications (Abstract). Society for Biological Therapy 1989 Annual Meeting, Williamsburg, VA, 1989.
179. Laguzza BC, Nichols CL, Briggs SL, et al. New antitumor monoclonal antibody-vinca conjugates. LY203725 and related compounds: Design, preparation and representative in vivo activity. *J Medicinal Chem* 1989;32:548-555.
180. Takahashi T, Yamaguchi T, Kitamura K, et al. Clinical application of monoclonal antibody-drug conjugates for immunotargeting chemotherapy of colorectal carcinoma. *Cancer* 1988;61:881-888.
181. Paul AR, Engstrom PF, Weiner LM, Steplewski A, Koprowski H. Treatment of advanced measurable or evaluable pancreatic carcinoma with 17-1A murine monoclonal antibody alone or in combination with 5-fluorouracil, adriamycin and mitomycin (FAM). *Hybridoma* 1986;5:S171-S174.
182. Spittler LE, delRio M, Khenigan A, et al. Therapy of patients with malignant melanoma using a monoclonal antimelanoma antibody-ricin A chain immunotoxin. *Cancer Res* 1987;47:1717-1723.
183. Byers VS, Rodvien R, Grant K, et al. Phase I study of monoclonal antibody-ricin A chain immunotoxin XomaZyme-791 in patients with metastatic colon cancer. *Cancer Res* 1989;49:6153-6160.
184. Order SE, Stillwagon GB, Klein JL, et al. Iodine 131 antiferritin, a new treatment modality in hepatoma: A radiation therapy oncology group study. *J Clin Oncol* 1985;3:1573-1582.
185. Roselli M, Schlom J, Gansow OA, et al. Comparative biodistributions of yttrium and indium labeled monoclonal antibody B72.3 in athymic mice bearing human colon carcinoma xenograft. *J Nucl Med* 1989;30:672-682.
186. Horan Hand P, Colcher D, Salomon D, Ridge J, Noguchi P, Schlom J. Influence of spatial configuration of carcinoma cell populations on the expression of a tumor associated glycoprotein. *Cancer Res* 1985;45:833-840.
187. Greiner JW, Fisher PB, Pestka S, Schlom J. Differential effects of recombinant human leukocyte interferons on cell surface antigen expression. *Cancer Res* 1986;46:4984-4990.
188. Matsui M, Nakanishi T, Noguchi T, Ferrone S. Synergistic *in vitro* and *in vivo* anti-tumor effect of daunomycin-anti-96kDa melanoma-associated antigen monoclonal antibody CL 207 conjugate and recombinant IFN- γ . *J Immunol* 1988;141:1410-1417.
189. Greiner JW, Horan Hand P, Noguchi P, Fisher P, Pestka S, Schlom J. Enhanced expression of surface tumor-associated antigens on human breast and colon tumor cells after recombinant human leukocyte alpha-interferon treatment. *Cancer Res* 1984;44:3208-3214.
190. Kantor J, Tran R, Greiner J, et al. Modulation of carcinoembryonic antigen mRNA levels in human colon carcinoma cells by recombinant human gamma interferon. *Cancer Res* 1989;49:2651-2655.
191. Guadagni F, Schlom J, Johnston WW, et al. Selective interferon-induced enhancement of tumor-associated antigens on a spectrum of freshly isolated human adenocarcinoma cells. *J Natl Cancer Inst* 1989;81:502-512.
192. Greiner JW, Guadagni F, Noguchi P, et al. Recombinant interferon enhances mono-

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sions *in vivo*. *Science* 1987;235:895-898.
193. Rosenblum MG, Lamki LM, Murray JL, Carlo DJ, Gutterman JU. Interferon-induced changes in pharmacokinetics and tumor uptake of ¹¹¹In-labeled antimelanoma antibody 96.5 in melanoma patients. *J Natl Cancer Inst* 1988;80:160-165.
194. Greiner JW, Guadagni F, Goldstein D, et al. Preclinical and phase IA/IB clinical studies demonstrating that interferon-gamma (IFN- γ) augments the level of tumor associated glycoprotein-72 (TAG-72) expression on human ovarian carcinoma cells (Abstract). 1989 Annual Meeting of the International Society for Interferon Research. Florence, Italy, 1989.
195. Eisenthal A, Cameron RB, Uppenkamp I, Rosenberg SA. Effect of combined therapy with lymphokine-activated killer cells, interleukin 2 and specific monoclonal antibody on established B16 melanoma lung. *Cancer Res* 1988;48:7140-7145.
196. Segal DM, Wunderlich JR. Targeting of cytotoxic cells with heterocrosslinked antibodies. *Cancer Invest* 1988;6:83-92.
197. Blumenthal RD, Sharkey RM, Snyder DJ, Hansen HJ, Goldenberg DM. Reduction of radioantibody-induced myelotoxicity in hamsters by recombinant interleukin-1. *Cancer Res* 1988;48:5403-5406.
198. Ohuchi N, Simpson J, Colcher D, Schlom J. Complementation of anti-CEA and anti-TAG-72 monoclonal antibodies in reactivity to human gastric adenocarcinomas. *Int J Cancer* 1987;40:726-733.
199. Estlin CD, Stevenson US, Plowman GD, et al. Recombinant vaccinia virus vaccine against the human melanoma antigen p97 for use in immunotherapy. *Proc Natl Acad Sci USA* 1988;85:1052-1056.
200. Morrison SL, Schlom J. Recombinant chimeric monoclonal antibodies. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Important advances in oncology*. Philadelphia: JB Lippincott, in press.
201. Hattt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. *Nature* 1989;342:76-78.
202. Mulligan RC, Berg P. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc Natl Acad Sci USA* 1981;78:2072-2076.
203. Southern PJ, Berg P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet* 1982;1:327-341.
204. Sagahan BG, Drai H, Saltzgarker-Muller J, et al. A genetically engineered murine-human chimeric antibody retains specificity for human tumor-associated antigen. *J Immunol* 1986;137:1066-1074.
205. Sun LK, Curtis P, Rakowicz-Szulczynska E, et al. Chimeric antibody with human constant regions and mouse variable regions directed against carcinoma-associated antigen 17-1A. *Proc Natl Acad Sci USA* 1987;84:214-218.
206. Fogler WE, Sun LK, Klinger MR, Ghraeyeb J, Daddona PE. Biological characterization of a chimeric mouse-human IgM antibody directed against the 17-1A antigen. *Cancer Immunol Immunother* 1989;30:43-50.
207. Whittle N, Adair J, Lloyd C, et al. Expression in COS cells of a mouse-human chimeric B72.3 antibody. *Protein Eng* 1987;1:499-505.
208. Hutzell P, Kashmiri S, Colcher D, et al. Generation and characterization of a recombinant/chimeric B72.3 (human γ 1). *Cancer Res* 1991;51:181-189.
209. Nishimura Y, Yokoyama M, Araki K, Veda R, Kudo A, Wanatabe T. Recombinant human-mouse chimeric monoclonal antibody specific for common acute lymphocytic leukemia antigen. *Cancer Res* 1987;47:999-1005.
210. Liu AY, Robinson R, Murray D, Ledbetter JA, Hellstrom I, Hellstrom KE. Production of a mouse-human chimeric monoclonal antibody to CD20 with potent Fc-dependent biologic activity. *J Immunol* 1987;139:3521-3526.
211. Liu AY, Robinson RR, Hellstrom KE, Murray ED, Chang CP, Hellstrom I. Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells. *Proc Natl Acad Sci USA* 1987;84:3439-3443.
212. Beidler CB, Ludwig JR, Cardenas J, et al. Cloning and high level expression of a chimeric antibody with specificity for human carcinoembryonic antigen. *J Immunol* 1988;141:4053-4060.
213. Hardman N, Gill LL, DeWinter RFJ, et al. Generation of a recombinant mouse-human chimeric monoclonal antibody directed against human carcinoembryonic antigen. *Int J Cancer* 1989;44:424-433.
214. Koga H, Kanda H, Nakashima M, Watanabe Y, Endo K, Watanabe T. Mouse-human chimeric monoclonal antibody to carcinoembryonic antigen (CEA): *in vitro* and *in vivo* activities. *Hybridoma* 1990;9:43-56.
215. Orlandi R, Gussow DH, Jones PT, Winter G. Cloning immunoglobulin variable domains

- for expression by the polymerase chain reaction. *Proc Natl Acad Sci USA* 1989; 86:3833-3837.
216. Colcher D, Milenic D, Roselli M, et al. Characterization and biodistribution of recombinant and recombinant/chimeric constructs of monoclonal antibody B72.3. *Cancer Res* 1989;49:1738-1745.
 217. Hale G, Clark MR, Marcus R, et al. Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet* 1988;17:1394-1399.
 218. Primus FJ, Pendurthi TK, Hutzell P, Kashmiri S, Callahan R, Schlom J. Chimeric B72.3 mouse-human (γ 1) antibody directs the lysis of tumor cells by lymphokine-activated killer cells. Submitted for publication.
 219. Mattes MJ. Biodistribution of antibodies after intraperitoneal or intravenous injection and effect of carbohydrate modification. *J Natl Cancer Inst* 1987;79:855-863.
 220. FitzGerald DJ, Waldmann TA, Willingham MC, Pastan I. Pseudomonas exotoxin-anti-TAC. Cell-specific immunotoxin active against cells expressing the human T cell growth factor receptor. *J Clin Invest* 1984;74:966-971.
 221. Kondo T, FitzGerald C, Chaudhary VK, Adhya S, Pastan I. Activity of immunotoxins constructed with modified *Pseudomonas* exotoxin A lacking the cell recognition domain. *J Biol Chem* 1988;263:9470-9475.
 222. Tan LK, Morrison SL. Antibody structure and antibody engineering. *Adv Drug Del Rev* 1988;2:129-142.
 223. Larson SM, Raubitschek A, Reynolds JC, et al. Comparison of bone marrow dosimetry and toxic effect of high dose I-131 labeled monoclonal antibodies administered to man. *Int J Radiat Appl Instrum* 1989;16:153-158.
 224. Huston JS, Levinson D, Mudgett-Hunter M, et al. Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci USA* 1988;85:5879-5883.
 225. Chaudhary VK, Queen C, Junghans RP, Waldmann TA, FitzGerald DJ, Pastan I. A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature* 1989;339:394-397.
 226. Colcher D, Bird R, Roselli M, et al. *In vivo* application of a recombinant single chain antigen binding protein. Submitted for publication.
 227. Huse WD, Sastry L, Iverson SA, et al. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 1989;246:1275-1281.
 228. Ward ES, Gussow D, Griffiths AD, Jones PT, Winter G. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature* 1989;341:544-546.
 229. Frankel ME, Gerhard W. The rapid determination of binding constants for antiviral antibodies by a radioimmunoassay. An analysis of the interaction between hybridoma proteins and influenza virus. *Mol Immunol* 1979;16:101-106.
 230. Sakahara H, Reynolds JC, Carrasquillo JA, et al. *In vitro* complex formation and biodistribution of mouse antitumor monoclonal antibody in cancer patients. *J Nucl Med* 1989;30:1311-1317.
 231. Roitt IM, Brostoff J, Male DK. *Immunology*. London: Gower Medical Publishing Ltd., 1985:5.2.

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Prostate-specific Membrane Antigen Expression in Normal and Malignant Human Tissues¹

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ABSTRACT

Prostate-specific membrane antigen is a type II membrane protein with folate hydrolase activity produced by prostatic epithelium. The expression of this molecule has also been documented in extraprostatic tissues, including small bowel and brain. In the present study, an extensive immunohistochemical analysis was performed on a panel of well-characterized normal and malignant human tissues to further define the pattern of prostate-specific membrane antigen (PSMA) expression.

Detectable PSMA levels were identified in prostatic epithelium, duodenal mucosa, and a subset of proximal renal tubules. A subpopulation of neuroendocrine cells in the colonic crypts also exhibited PSMA immunoreactivity. All other normal tissues, including cerebral cortex and cerebellum, had undetectable levels of PSMA. Thirty-three of 35 primary prostate adenocarcinomas and 7 of 8 lymph node metastases displayed tumor cell PSMA immunostaining. Eight of 18 prostate tumors metastatic to bone expressed PSMA. All of the other nonprostatic primary tumors studied had undetectable PSMA levels. However, intense staining was observed in endothelial cells of capillary vessels in peritumoral and endotumoral areas of certain malignancies, including 8 of 17 renal cell carcinomas, 7 of 13 transitional cell carcinomas, and 3 of 19 colon carcinomas.

Extraprostatic PSMA expression appears to be highly restricted. Nevertheless, its diverse anatomical distribution implies a broader functional significance than previously suspected. The decrease in PSMA immunoreactivity noted in advanced prostate cancer suggests that expression of this molecule may be linked to the degree of tumor differentia-

tion. The neoexpression of PSMA in endothelial cells of capillary beds in certain tumors may be related to tumor angiogenesis and suggests a potential mechanism for specific targeting of tumor neovasculature.

INTRODUCTION

PSMA³ is a *M*_r 100,000 type II membrane protein consisting of 750 amino acids (1, 2). Although PSMA exhibits *in vitro* neuropeptidase activity (3), its function *in vivo* has not been fully elucidated. The PSMA gene is located on chromosome 11 (4, 5). To date, two molecular forms of the protein, designated PSMA and PSMA', have been identified (6). Monoclonal antibody 7E11-C5 (designated CYT 351) recognizes an intracellular epitope of PSMA (7) and has been previously utilized for its immunohistochemical detection (6, 8). In addition, a radioimmunoconjugate of the 7E11-C5 antibody (designated CYT 356) is in use as an imaging agent for prostate tissues (7, 9-12). The clinical utility of PSMA as a marker for prostate disease depends on the pattern of its expression *in vivo*. An extensive immunohistochemical evaluation was performed to further characterize the pattern of PSMA expression in normal human tissues. A selected panel of primary and metastatic prostate carcinomas was also evaluated as well as a representative cohort of other epithelial malignancies, including renal cell carcinoma, bladder transitional cell carcinoma, and colon carcinoma.

MATERIALS AND METHODS

Tissues. Normal and neoplastic formalin-fixed, paraffin-embedded tissue samples were obtained from the Department of Pathology at the Memorial Sloan-Kettering Cancer Center. Thirty-five primary prostate adenocarcinoma specimens were evaluated as well as 8 metastases to lymph nodes and 18 metastases to bone. Table 1 summarizes the clinical characteristics of these tumors in terms of pathological stage and prior treatment. Additional primary tumors included 17 renal cell carcinomas, 13 bladder transitional cell carcinomas, and 19 colon adenocarcinomas.

Antibodies. Mouse monoclonal antibody CYT-351 (clone 7E11-C5) (Cytogen, Princeton, NJ) was used as the primary antibody. This clone is derived from the original hybridoma reported by Horoszewicz *et al.* (7). Secondary antibodies consisted of biotinylated horse anti-mouse polyclonal IgG (Vector Laboratories, Inc., Burlingame, CA). The proper concentration of each reagent was determined by titration experiments prior to staining.

Immunohistochemistry. An avidin-biotin peroxidase method was used. Sections were deparaffinized, and endogenous peroxidase activity was blocked in 1.0% hydrogen perox-

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³ The abbreviation used is: PSMA, prostate-specific membrane antigen.

Table 1 Primary prostate tumors: stage and prior treatment

Stage	ADT/				ADT/RT/				Totals
	ADT ^a	RT	Chemo	RT	Chemo	Chemo	None		
P ₂ N ₀	1						14		
P ₁ N ₀	1	1					14		35
P ₃ N ₀	1						3		
N+	2						6		8
M+	3	2	1	5	3	2	2		18

^aADT, androgen deprivation therapy; RT, radiation therapy; Chemo, chemotherapy.

ide in PBS for 15 min. Sections were immersed in boiling 0.01% citric acid (pH 6.0) for 15 min to enhance antigen retrieval and allowed to cool. In some cases, endogenous biotin was blocked with an avidin-biotin blocking kit (Vector Laboratories). Normal horse blocking serum (Organon Teknica Corp., West Chester, PA) at a 1:10 dilution in 2% PBS-BSA (Sigma Chemical Co., St. Louis, MO) was applied for 30 min to minimize background staining. Primary antibody at 2 µg/ml in 2% PBS/BSA was applied after suction removal of horse serum, and sections were incubated overnight in a wet chamber at 4°C. Sections were washed and biotinylated secondary antibodies were applied for 30 min (1:500 dilution). Sections were washed and avidin-biotin peroxidase complexes (Vector Laboratories) diluted 1:25 in PBS were applied for 30 min. Sections were then immersed in a solution of 0.05% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide in 0.5% Triton X-100-PBS to accomplish the chromogen reaction. After extensive washing, sections were counterstained with hematoxylin, dehydrated, and mounted. Cases were considered positive if at least 20% of the malignant component demonstrated immunoreactivity. Positive control antibodies to normal antigen components present in specific cell types included CD45 (DAKO Corp., Carpinteria, CA) diluted 1:500 in 2% PBS-BSA and chromogranin (DAKO Corp.) diluted 1:20,000 in 2% PBS-BSA. Negative controls were conducted by substitution of primary antibodies with non-immune serum.

RESULTS

Table 2 summarizes immunoreactivities identified in the normal tissues studied. In normal and hyperplastic prostate glands, staining was either weak and luminal or absent. In several tissues, the immunohistochemical procedure routinely utilized was modified to include blocking of endogenous biotin to avoid false-positive reactions. Renal tubules, initially noted to display intense cytoplasmic staining, exhibited identical reaction patterns with class-matched primary antibody substitution and negative controls (Fig. 1a). Blocking of endogenous biotin abolished the background cytoplasmic staining and revealed immunoreactivity that was reproducibly restricted to a subset of proximal tubules (Fig. 1b). A similar situation was encountered in the gastrointestinal tract, with intense staining of the duodenal and colonic mucosa. Blocking of endogenous biotin revealed persistent immunoreactivity limited to the duodenal brush border (Fig. 1c). Rare cells in the deepest portions of the colonic crypts were immunoreactive (Fig. 1d); these had a morphology and distribution similar to those of chromogranin-positive cells

Table 2 PSMA expression in normal tissues

Tissue	PSMA
Genitourinary organs	
Kidney	
Glomeruli	-
Tubules	+
Bladder	
Transitional epithelium	-
Smooth muscle	-
Prostate	
Epithelium	+
Stroma	-
Testis	-
Cervix	-
Breast	-
Digestive system	
Parotid	-
Stomach	-
Duodenum	+
Ileum	-
Colon	+
Liver	-
Pancreas	-
Hematological system	
Lymph node	-
Bone marrow	-
Skin	-
Skeletal muscle	-
Endocrine organs	
Thyroid	-
Adrenal	-
Cortex	-
Medulla	-
Pancreatic islets	-
Nervous system	
Frontal cortex	-
Cerebellum	-
Eye	-
Peripheral ganglion	-

in serial sections (data not shown), implying a possible neuroendocrine origin.

Table 3 summarizes immunoreactivities identified in the tumors studied. Significant PSMA expression was detectable in 33 of 35 primary prostate tumors. The pattern of staining varied with the degree of differentiation, with the most intense and homogeneous reactivity located at the luminal site of the glands in well-differentiated tumors (Fig. 2a). Immunoreactivity was more heterogeneous in less well-differentiated lesions (Fig. 2b). Considerable heterogeneity of expression within the same tumor was noted in most cases. No immunoreactivity was present in prostatic stromal elements, including blood vessels.

Similarly, seven of eight prostate carcinomas metastatic to lymph nodes expressed detectable PSMA levels (Fig. 2c). In the majority of cases, the staining pattern was reminiscent of that observed in poorly differentiated primary tumors, without any noticeable subcellular orientation. In one case, pseudogland formation was present with intense reactivity at the luminal site. Staining within a metastatic deposit was less heterogeneous than that in the primary tumors, with cells virtually all positive or all negative. Lymphoid elements did not exhibit immunoreactivities. The 18 osseous metastases of prostate carcinoma were divided between cases with and without detectable PSMA ex-



Fig. 1 PSMA expression in selected normal tissues. Granular cytoplasmic staining of epithelial cells of proximal renal tubules in nonbiotin-blocked tissue sections (a). Blocking of endogenous biotin removed the nonspecific cytoplasmic staining and revealed a persistent immunoreactivity restricted to the luminal site in a subset of proximal renal tubules (b). Biotin-blocked section of duodenum showed strong PSMA immunoreactivity at the mucosal brush border (c). PSMA immunostaining of a neuroendocrine cell in a colonic crypt (d). a, b, and d. $\times 400$; c, $\times 200$.

Table 3 PSMA expression in tumor tissues

Carcinoma	No. studied	No. immunopositive	
		Tumor cells	Neovascularity
Prostate, primary	35	33	0
Prostate, metastatic			
Lymph node	8	7	
Bone	18	8	
Renal cell	17	0	8
Bladder transitional cell	13	0	7
Colon	19	0	3

pression. The eight cases with immunostaining demonstrated considerable heterogeneity within each specimen (Fig. 2d). No staining of osseous or hematopoietic elements was observed.

None of the 17 renal cell carcinomas, 13 bladder transitional cell carcinomas, and 19 colon adenocarcinomas evaluated showed detectable PSMA levels in the tumor cells. Stromal components were similarly negative, except for some blood vessels. Capillary endothelial cell immunoreactivity restricted to the region of the tumor was noted in 8 of 17 renal cell carcinomas (Fig. 3a), in 7 of 13 transitional cell carcinomas (Fig. 3b), and in 3 of 19 colon tumors (Fig. 3, c and d). Capillaries located in normal tissue adjacent to the tumors were not immunoreactive. Considerable heterogeneity of expression was evident, with virtually all peritumor capillaries positive in some cases and only a few capillaries positive in others. Blocking of endogenous biotin did not change this result, and it was not seen in control sections utilizing a class-matched primary antibody.

DISCUSSION

The present study is supportive of previous evaluations of PSMA expression in normal tissues, with several distinctions. Expression of PSMA by a subset of renal tubules cannot be

regarded as artifactual, since controls in biotin-blocked sections confirm this finding. The identification of rare PSMA-expressing cells in the colonic crypts represents a new finding. The morphology and immunohistochemical characteristics of these cells indicate a neuroendocrine origin. The significance of this finding is not clear, but it parallels the recent report by Carter *et al.* (3) of a carboxypeptidase involved in central nervous system glutamate metabolism with remarkable homology to PSMA. The finding of PSMA expression in the duodenum is consistent with the previous detection of PSMA mRNA transcripts in small bowel (13) and of PSMA in small bowel protein extracts (14). Additionally, monoclonal antibody 7E11-C5 has recently been shown to precipitate a molecule with folate hydrolase activity from prostate carcinoma cell line extracts (15), a finding which parallels the known high level of folate hydrolase activity in duodenal mucosa. Folate hydrolase is a carboxypeptidase and, like the brain enzyme, liberates glutamate as a reaction product. The possible function of PSMA as a folate hydrolase in the duodenum and in the prostate is currently under investigation. PSMA mRNA transcripts were also identified in central nervous system (13). However, immunohistochemically detectable PSMA expression was not seen in either cerebral cortex or cerebellum in the present study. This may represent expression of the alternatively spliced molecular form of PSMA (PSMA') lacking the epitope recognized by 7E11-C5 or expression at a specific brainstem or ganglionic locus not analyzed.

The present study confirms results from previous analyses with respect to the immunohistochemical detection of PSMA expression in primary and metastatic prostate cancer. Horoszewicz *et al.* (7) described immunoreactivity in frozen prostate tissues, including nine of nine normal prostates, nine of nine primary prostatic carcinomas, and two of two lymph node metastases. Lopes *et al.* (8) compared staining patterns of 7E11-C5 and the radionuclide-labeled immunoconjugate CYT-356 in frozen prostate tissues. They noted immunohistochemical

Fig. 2 PSMA expression in prostatic carcinoma. Intense PSMA immunoreactivity in the glandular epithelium located mainly at the luminal site of a well-differentiated primary tumor (a). More homogeneous cytoplasm and membrane immunostaining of a poorly differentiated primary tumor (b). PSMA expression by tumor cells of lymph node metastasis. Note the absence of staining in lymphoid elements (c). Osseous metastasis showing a heterogeneous pattern of PSMA immunoreactivity (d). a-c, $\times 200$; d, $\times 400$.

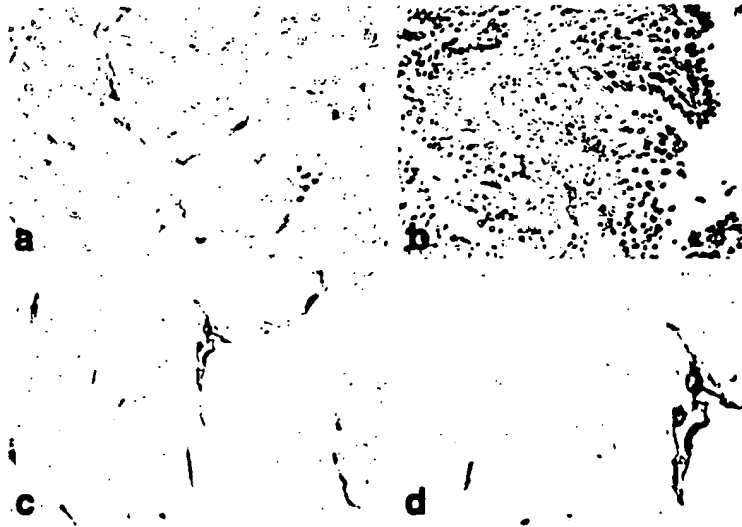
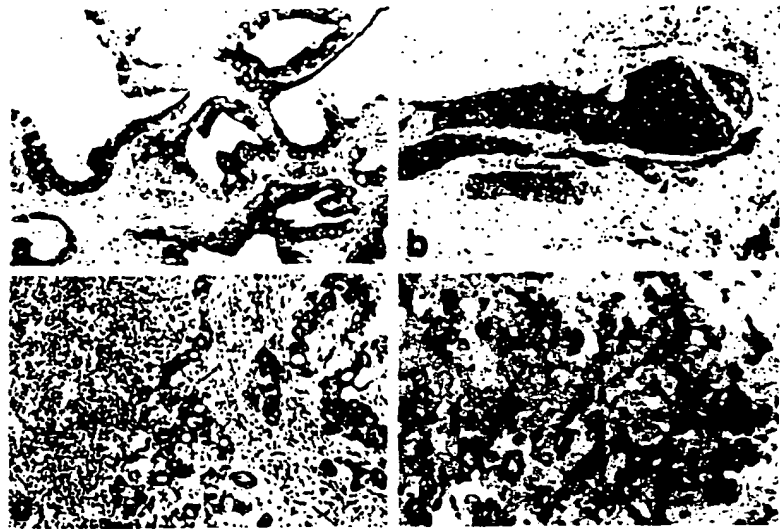


Fig. 3 PSMA expression by neovascular capillary endothelial cells in peritumoral areas of selected primary epithelial malignancies. Renal cell carcinoma (a), transitional cell carcinoma of the urinary bladder (b), and colonic adenocarcinoma (c and d). Note the intense immunostaining of endothelial cells, whereas tumor cells had undetectable PSMA levels. a and d, $\times 400$; b and c, $\times 200$.

detection of PSMA by 7E11-C5 in an unspecified number of normal prostates and in 10 primary prostatic carcinomas. Wright *et al.* (9) found PSMA immunoreactivity in all normal prostates analyzed, 157 of 165 primary tumors, and 72 of 79 lymph node metastases.

With respect to prostate cancer metastatic to bone, Wright *et al.* (9) noted that all of the seven cases examined expressed PSMA. This is at variance with the current study, in which PSMA expression could be detected in only 8 of 18 osseous metastases (Table 2). This difference may be due to sample size or it may be related to the degree of differentiation and extensive

prior treatment (androgen deprivation, radiation, chemotherapy) of the lesions analyzed. It is also possible that some bone metastases express the alternatively spliced form of PSMA (PSMA') lacking the epitope recognized by 7E11-C5. Additionally, although down-regulation of PSMA mRNA expression in response to androgen has been demonstrated *in vitro*, with the greatest expression noted at castrate levels of androgen (13), PSMA detection in the present study was lowest in the group of patients failing androgen deprivation. These patients represent those with tumor progression to osseous metastases despite hormonal manipulation. These findings support the hypothesis

that the interaction of tumor with the metastatic site has an effect on tumor phenotype (16).

PSMA expression was not detected in a variety of primary epithelial tumors. The lack of PSMA in renal cell carcinomas is of interest, in view of its expression in a subset of proximal tubules. It is known that renal cell carcinomas, specifically clear cell and granular cell carcinomas, are derived from proximal epithelial cells. The undetectable PSMA levels in the renal cell carcinomas analyzed may be due to the loss of PSMA during malignant transformation. Alternatively, the lack of PSMA in the renal tumors studied may indicate that they are derived from cells not displaying the PSMA-positive phenotype. Similarly, the cells which express PSMA in colonic crypts are of neuroendocrine derivation. Since these cells are not the precursors of colonic adenocarcinomas, the lack of PSMA staining in tumor cells from these neoplasms is not unexpected.

An important finding of the present study is the novel demonstration of PSMA expression by neovascular capillary endothelium in the peritumoral areas of a variety of epithelial malignancies. The significance of this finding in terms of the function of PSMA is presently unclear; however, it may have therapeutic implications. Humanized anti-PSMA antibodies could be used to deliver a variety of agents aimed at destroying neovasculature, ranging from conventional cellular toxins to peptide-based prodrug activators. Additionally, analysis with RNase protection techniques has demonstrated the presence of PSMA mRNA in both healthy and diseased prostate tissue (13). Further understanding of the PSMA gene's control mechanisms may be useful in the development of promoter-driven gene therapy for both benign and malignant prostate diseases.

In summary, PSMA appears to be highly expressed in normal prostate tissue as well as primary and nodally metastatic prostate cancer. In the present study, 40% of prostate cancers metastatic to bone expressed PSMA. Examination of normal tissues revealed PSMA expression in prostate epithelium, duodenal mucosa, a subset of renal tubules, and certain neuroendocrine cells in colonic crypts. Carcinomas arising in the bladder, kidney, and colon do not appear to express PSMA. PSMA expression by peritumor capillaries must be examined in other malignancies to establish the range of this phenomenon.

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REFERENCES

1. Abdel-Nabi, H., Wright, G. L., Gulfo, J. V., Petrylak, D. P., Neal, C. E., Texter, J. E., Begun, F. P., Tyson, I., Heal, A., Mitchell, E., Purnell, G., and Harwood, S. J. Monoclonal antibodies and radioimmunoconjugates in the diagnosis and treatment of prostate cancer. *Semin. Urol.*, 10: 45-54, 1992.
2. Israeli, R. S., Powell, C. T., Fair, W. R., and Heston, W. D. W. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.*, 53: 227-230, 1993.
3. Carter, R. E., Feldman, A. R., and Coyle, J. T. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc. Natl. Acad. Sci. USA*, 93: 749-753, 1996.
4. Rinker-Schaeffer, C. W., Hawkins, A. L., Su, S. L., Israeli, R. S., Griffin, C. A., Isaacs, J. T., and Heston, W. D. W. Localization and physical mapping of the prostate-specific membrane antigen (PSMA) gene to human chromosome 11. *Genomics*, 30: 105-108, 1995.
5. Leek, J., Lench, N., Maraj, B., Bailey, A., Carr, I. M., Andersen, S., Cross, J., Whelan, P., MacLennan, K. A., Meredith, D. M., and Markham, A. F. Prostate-specific membrane antigen: evidence for the existence of a second related human gene. *Br. J. Cancer*, 72: 583-588, 1995.
6. Su, S. L., Huang, I-P., Fair, W. R., Powell, C. T., and Heston, W. D. W. Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. *Cancer Res.*, 55: 1441-1443, 1995.
7. Horoszewicz, J. S., Kawinski, E., and Murphy, G. P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. *Anticancer Res.*, 7: 927-936, 1987.
8. Lopes, A. D., Davis, W. L., Rosenstraus, M. J., Uveges, A. J., and Gilman, S. C. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from anti-prostate monoclonal antibody 7E11-C5. *Cancer Res.*, 50: 6423-6429, 1990.
9. Wright, G. L., Jr., Haley, C., Beckett, M. L., and Schellhammer, P. F. Expression of prostate-specific membrane antigen in normal, benign and malignant prostate tissues. *Urol. Oncol.*, 1: 18-28, 1995.
10. Sanford, E., Grzonka, R., Heal, A., Helal, M., Persky, L., and Tyson, I. Prostate cancer imaging with a new monoclonal antibody: a preliminary report. *Ann. Surg. Oncol.*, 1: 400-404, 1994.
11. Kahn, D., Williams, R. D., Seldin, D. W., Libertino, J. A., Hirschhorn, M., Dreicer, R., Weiner, G. J., Bushnell, D., and Gulfo, J. Radioimmunosintigraphy with ¹¹¹indium labeled CYT-356 for the detection of occult prostate cancer recurrence. *J. Urol.*, 152: 1490-1495, 1994.
12. Babaian, R. J., Sayer, J., Podoloff, D. A., Steelhammer, L. C., Bhadkamkar, V. A., and Gulfo, J. V. Radioimmunosintigraphy of pelvic lymph nodes with ¹¹¹indium-labeled monoclonal antibody CYT-356. *J. Urol.*, 152: 1952-1955, 1994.
13. Israeli, R. S., Powell, C. T., Corr, J. G., Fair, W. R., and Heston, W. D. W. Expression of the prostate-specific membrane antigen. *Cancer Res.*, 54: 1807-1811, 1994.
14. Troyer, J. K., Beckett, M. L., and Wright, G. L., Jr. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int. J. Cancer*, 62: 552-558, 1995.
15. Pinto, J., Suffoletto, B., Berzin, T., Qiao, C., Lin, S., Tong, W., and Heston, W. D. W. Identification of a membrane-bound pteroyl poly gamma-glutamyl carboxypeptidase (folate hydrolase) that is highly expressed in human prostatic carcinoma cells (Abstract 2862). *FASEB J.*, 10: A496, 1996.
16. Thalmann, G. N., Ploutarchos, E. A., Chang, S-M., Zhou, H. E., Kim, E. E., Hopwood, V. L., Pathak, S., von Eschenbach, A. C., and Chung, L. W. K. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res.*, 54: 2577-2581, 1994.

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PROCEEDINGS OF THE FIRST INTERNATIONAL CONFERENCE ON NEOADJUVANT HORMONAL THERAPY FOR PROSTATE CANCER

Guest Editors: WILLIAM R. FAIR, M.D., and MARC B. GARNICK, M.D., F.A.C.P.

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CME ISSUE

PROCEEDINGS OF THE FIRST INTERNATIONAL CONFERENCE ON NEOADJUVANT HORMONAL THERAPY FOR PROSTATE CANCER

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CHARACTERIZATION AND GLUTAMYL PREFERRING CARBOXYPEPTIDASE FUNCTION OF PROSTATE SPECIFIC MEMBRANE ANTIGEN: A NOVEL FOLATE HYDROLASE

WARREN D. W. HESTON

ABSTRACT

We have cloned the gene encoding the prostate-specific membrane (PSM) antigen, which is recognized by the 7E11C-5 antibody. The antigen is strongly expressed in prostate cancer, and the antibody has been approved for use as an imaging agent for detection of prostatic cancer metastasis. The gene was unique and encoded a type II membrane protein. The only clue to its potential function was found in the cDNA coding sequences from 1250 to 1700, which had a modest but significant homology with transferrin-receptor, demonstrating a 54% homology of nucleic acid sequence.

In comparing the mRNA obtained from normal prostate with that obtained from cancerous or lymph node carcinoma of the prostate (LNCaP) cells, normal cells produced a shorter alternative spliced species that encoded a cytosolic form of the protein, and not a membrane protein. It appeared that, as the prostatic cells became cancerous, there was a nearly 100-fold difference in expression of the ratio of the messages encoding the 2 forms, with the cytosolic form (PSM') predominant in normal cells and the membrane form (PSM) predominant in cancer cells. The other tissue in which the membrane antigen form of PSM is highly expressed is the membrane brush border of the small intestine of the proximal, but not distal, small intestine. This is the location of a unique membrane form of a folate hydrolase. This membrane folate hydrolase and its location are necessary in human nutrition because humans require folate, and the folate in foods is poly-gamma-glutamated. Polyglutamated folates cannot be taken into the cells by folate-transporter systems. The ability to take up folate from foods requires the membrane folate hydrolase to sequentially remove the gamma-linked glutamates, freeing folate that can then be transported. PSM antigen has a similar folate hydrolase activity.

Others have reported finding an enzyme in the rat brain that functions as an alpha-neurocarboxypeptidase and acts on the abundant brain peptide *N*-acetylasparylglutamate to generate glutamate and *N*-acetylaspartate. The 3'-end of the rat brain enzyme had 84% sequence homology with PSM antigen. Because this enzyme liberates glutamate in the brain, the enzyme is considered to have regulatory activity related to glutamate receptors. Current investigations are underway to determine whether glutamate receptors are present in prostate.

Thus, PSM antigen is a unique folate hydrolase-carboxypeptidase that can release glutamate with either gamma- or alpha-linkage. Its enzymatic activity raises a number of questions for consideration. In the normal prostate where the protein is intracellular, is PSM' antigen keeping folate in nonglutamated forms? If so, folate should be able to readily diffuse out of prostate cells, making the prostate gland an organ at risk for localized folate deficiency and carcinogenesis. In prostate tumor cells, with the enzyme outside of the cell, can PSM antigen be used for the activation of cytotoxic prodrugs? © 1997 by Elsevier Science Inc. All rights reserved. UROLOGY 49 (Suppl 3A): 104-112, 1997.

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In this article, I will provide an overview of the discovery of a function for prostate specific membrane (PSM) antigen. PSM antigen is a novel and highly expressed gene from the human prostate that is recognized by the mouse monoclonal antibody 7E11C-5. The cloning of this gene was accomplished using degenerate polymerase chain reaction (PCR) on an LNCaP cell cDNA library

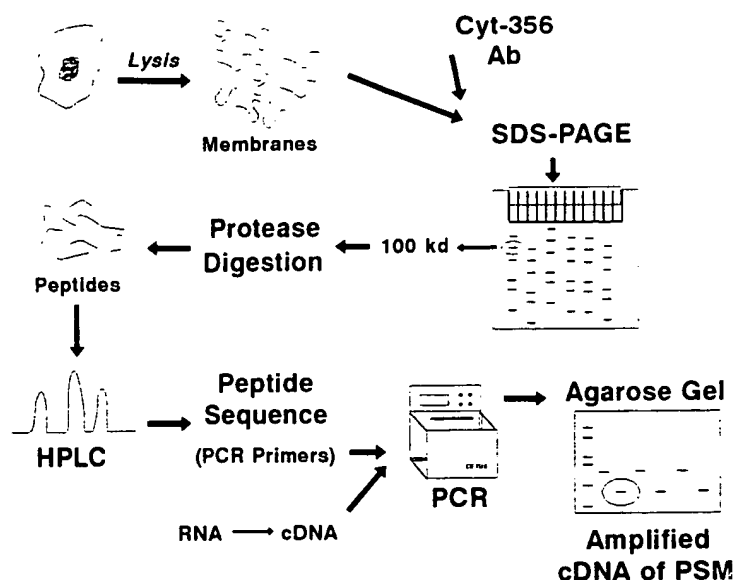


FIGURE 1. The procedures used in the cloning of the prostate specific membrane antigen.

after obtaining a sequence analysis of peptide fragments of PSM antigen. LNCaP cells are a human prostate cell line that maintains the known features of prostate cancer, especially the expression of biomarkers such as prostate specific antigen (PSA) and prostatic acid phosphatase (PAP).

Horoszewicz and his colleagues were responsible for the development of the unique cell line LNCaP.¹ Realizing the importance of being one of the first cell lines to maintain a high level of expression of known prostate biomarkers, Horoszewicz isolated the membranes from the LNCaP cells, injected them into Balb/c mice, and generated a monoclonal antibody (7E11C-5). This antibody exhibited a striking specificity for human prostate tissue, be it normal, benign, or malignant.^{1,2} The patent rights to this antibody were sold to the Cytogen Corp. (Princeton, NJ), which has focused on developing diagnostic imaging agents for cancer. Cytogen generously provided us with the 7E11C-5 monoclonal antibody for our research investigations.

The monoclonal antibody exhibited a very desirable characteristic, that is, it had the ability to serve as a specific immunoprecipitating agent of PSM antigen after the antigen had been extracted in a soluble form from the LNCaP cell membranes with the aid of a nonionic detergent. Following immunoprecipitation, the PSM antigen was resolubilized and run on sodium dodecyl sulfate (SDS) gel electrophoresis, the 100-kDa protein segment extracted from the gel, proteolytically digested to smaller fragments, fractionated by HPLC, and the

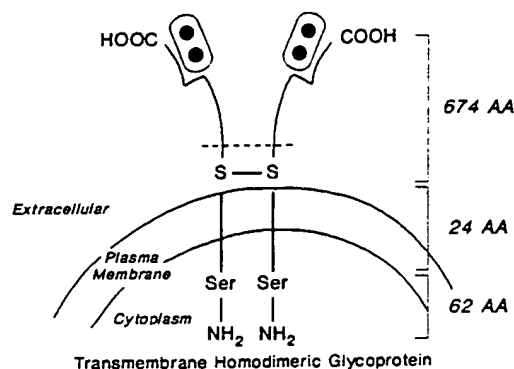
most pure fractions sequenced. These sequences could then be used to find the message encoding the gene from the LNCaP cDNA by degenerate PCR amplification. A 1.1-kilobase (kb) band was found that possessed the sequences of the predicted amino acids. The 1.1-kb probe was used to identify the full-length clone 55A, which was 2.65 kb in size. A graphical summary of the procedure used to clone the gene is outlined in Figure 1.³

The sequence of the cDNA was unique. The 2653-base pair PSM antigen cDNA predicted a 750-amino acid protein sequence. Coding sequences from 1250 to 1700 had modest homology with transferrin-receptor sequences and demonstrated a 54% homology of nucleic acid sequence overall.³ Computer analysis of the sequence predicted a protein that was a type II membrane protein (Fig. 2). Type II membrane proteins are unusual in their membrane orientation, in that the amino-terminus of the protein is intracellular and a fairly short sequence, while the extracellular portion contains a fairly large carboxyl-terminus, in this case a 19-amino acid intracellular region, a 24-amino acid membrane-spanning region, and a 707-amino acid extracellular domain.³ In addition to the modest sequence homology with the transferrin receptor, it should be noted that the transferrin receptor is also a type II membrane protein. In searching for the function of a gene, it was hoped that the sequence data would be related to other known genes that have an established function. Such was not the case for PSM antigen, as the sequence was totally unique except for the noted

FIGURE 2. The nucleotide sequence of the PSM antigen cDNA as well as the amino acid sequence of the encoded protein.

nine phenylalanine, that functions in enabling the internalization of the receptor after it binds iron-loaded transferrin. This internalization amino acid sequence is absent from the intracellular fraction of the PSM antigen, casting doubt on PSM antigen serving a transport function. Western blot analysis of the transferrin receptor suggests that it exists primarily as a dimer. The initial characterization

Transferrin Receptor (TfR)



PSM Antigen

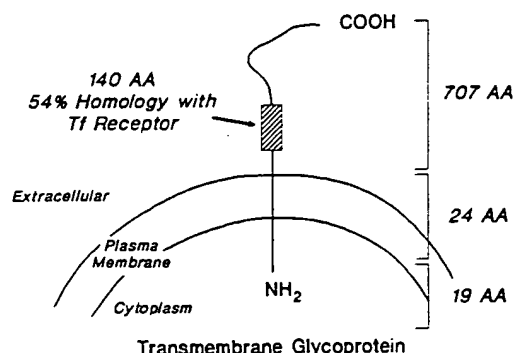


FIGURE 3. The structure of the transferrin receptor and the PSM antigen.

of PSM antigen in LNCaP cells would be more consistent with a monomeric form, although in prostate tissue extracts a larger molecular weight species has been observed and PSM antigen could possibly exist as a dimer. Regardless of how similar or dissimilar PSM antigen and the transferrin receptor are, the question of whether PSM antigen will bind to transferrin or transferrin-like molecules has not been addressed and is currently under study.

Could it be that the internalization sequence was missed because PSM antigen was isolated from a tumor cell and the tumor cell makes a different protein than the normal cell? Sequencing the cDNA generated from prostate tissue revealed that there was a difference at the 5' end, in the region of the amino-terminus where we would expect to see the internalization. However, examination of the difference did not provide a transferrin receptor-like internalization signal. Instead, normal tissue expressed an alternative spliced fragment smaller than that found in the LNCaP cells, which

was designated PSM' antigen.⁴ Based on computer analysis of PSM' antigen's structure, the spliced variant found in normal tissue was likely to be a cytosolic protein and definitely not a transport protein (Fig. 4). It was not possible to use the Cytogen antibody (CYT-356/7E11C-5) to determine the relative expression of the alternatively spliced form by immunohistochemistry because the monoclonal antibody that was used in the initial cloning only recognizes the intracellular region of the PSM molecule (Fig. 4).⁵ To circumvent the need for an antibody, an RNase protection assay was used to distinguish between the two forms. The results of the RNase protection assay revealed that there was 5 times more PSM mRNA than PSM' mRNA expressed in RNA extracted from tumors, about equal expression of both forms in RNA extracted from benign prostatic hyperplasia (BPH) tissue, and one-fifth to one-tenth as much PSM as PSM' expressed in RNA extracted from normal prostatic tissue. Thus, not only were there alternatively spliced variants of the PSM antigen being produced, but it appeared that as the cells became cancerous, there was a nearly 100-fold difference in expression of the ratio of the messages encoding the two forms.⁴ It is intriguing that this protein has such totally different locations between normal and cancerous prostatic cells. This difference in cellular location has to have an impact on the functioning of this protein (Fig. 4).

Another interesting aspect of the expression of this protein is that the mRNA encoding the message is upregulated by the absence of androgens. Indeed, androgens downregulate expression of the PSM message in the LNCaP cells.⁶ This observation was confirmed in patients, as Wright and colleagues observed an increase in expression of PSM antigen in patient specimens following hormone deprivation by immunohistochemistry.⁷ This contrasts with PSA, which is downregulated in expression by androgen deprivation. This emphasizes the potential utility of PSM antigen as a marker even in patients who are androgen deprived.⁷

The question remains: what is the function of PSM antigen? Most type II membrane proteins are either transport proteins, binding proteins, or proteases/hydrolases. Transport proteins seemed not to be likely. In considering the potential activity of PSM antigen, consideration of its strong expression in the proximal small intestine needed to be taken into account. Troyer *et al.*, using Western analysis with the 7E11C-5 antibody, observed that PSM was strongly expressed in the small intestine.⁸ Israeli *et al.* previously had observed a strong expression in RNA extracted from the small intestine.⁶ Silver *et al.* performed immunohistochemical analysis of tissues and found that PSM antigen

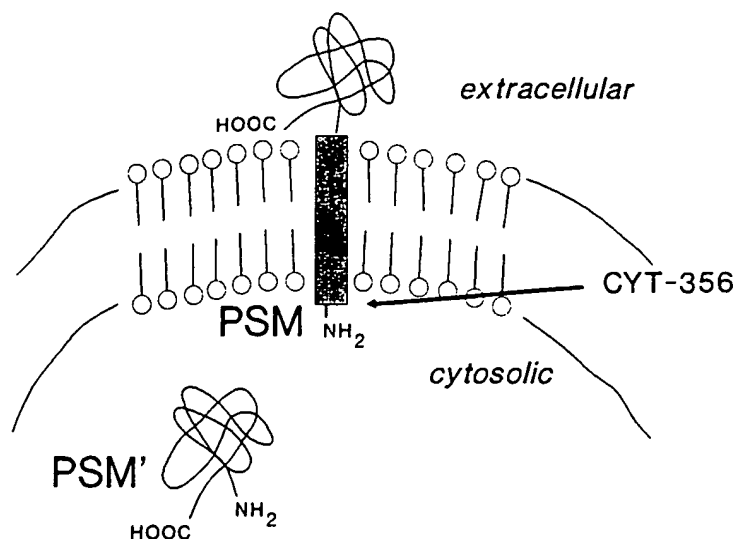


FIGURE 4. The proposed difference between the alternative spliced forms of PSM and PSM'. The region absent from PSM' is the amino-terminus fraction that would encode for a type II membrane protein. This amino-terminus region is recognized by the 7E11C-5 monoclonal antibody.

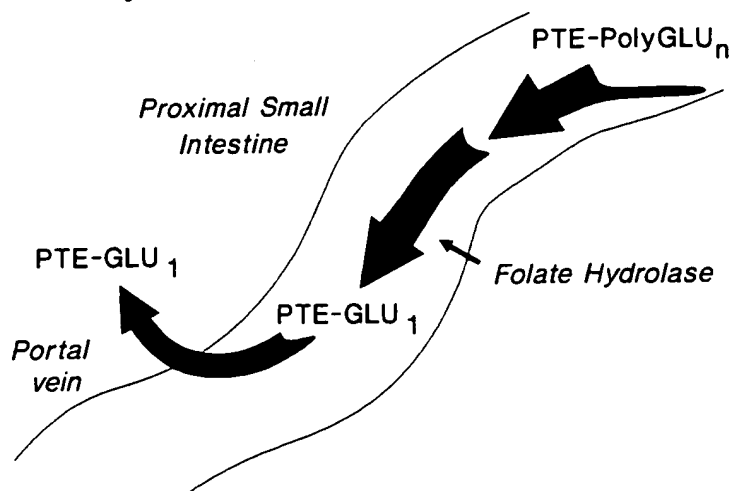
was strongly expressed in the brush border membrane of the proximal small intestine, but not in the distal small intestine.¹⁰ This location is consistent with a unique membrane hydrolase that releases the terminal gamma-linked glutamates from folate, folate hydrolase (Fig. 5).¹⁰ Pinto *et al.* found that LNCaP prostate cancer cells strongly express membrane folate hydrolase activity similar to that of the proximal intestinal unique brush border membrane folate hydrolase.¹¹ Pinto and colleagues further demonstrated that they could immunopurify this activity with the aid of the 7E11C-5 antibody. This raised the question of whether PSM antigen is membrane folate hydrolase, or whether the folate hydrolase activity was a contaminant carried into the immunoprecipitate. These investigators then transfected PC-3 cells with PSM antigen. Only the cells transfected with the PSM antigen had the membrane folate hydrolase activity, proving that they are one and the same.¹¹

Folates are normally polyglutamated following introduction into the cell. Polyglutamation serves to trap the folate inside the cell. Polyglutamated forms of folate have been found to be the preferred substrate for most of the folate-requiring reactions with most demonstrating a higher affinity for the folate-requiring enzymes. Folate hydrolase would be expected to reverse the degree of polyglutamation and permit the folate to be in a form that could easily diffuse out of the cell, putting the prostate at risk to become easily deficient in folate. Folate deficiencies are considered to have the po-

tential to be carcinogenic.¹² Of the folate-requiring enzymes, the enzyme methionine synthetase does not prefer the polyglutamated form as a substrate, and it may be that in the prostate the folate hydrolase serves to keep the folate in a nonpolyglutamated form for use with methionine synthetase. Methionine synthetase requires both folate and vitamin B-12 and generates methionine from homocysteine. Folate is important in cell reactions involving one carbon transfers. Methionine is important for use in methylation reactions such as nucleic acid methylation and for polyamine synthesis.¹²⁻¹⁴ The potential interrelationships of these activities and folate hydrolase in the prostate are being investigated.

If it is established that the folate found in prostate cells is not polyglutamated because of PSM' folate hydrolase activity, and that the prostate is susceptible to microenvironmental folate deficiencies, it could provide a rationale as to why there is such a high worldwide incidence of microscopic or incidental carcinoma of the prostate. Would folate supplementation reduce the incidence of microfocal prostate cancers? In cancer, the ratio of PSM' to PSM antigen changes so that more of the mRNA codes for the expression of the membrane-bound form in which the enzymatic activity would be outside of the cell. Is this serving to act as a binding protein for folate to try and help capture circulating folate? Is the membrane antigen helping to retrieve polyglutamated folate from surrounding dead and dying cells?

Dietary Folate Must Be Digested



Pteroylpentaglutamate

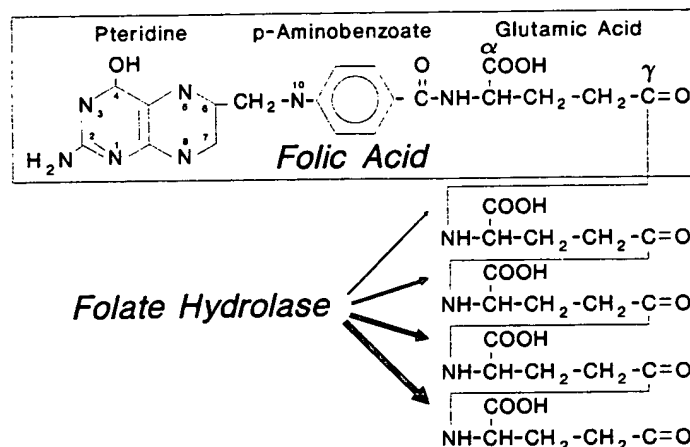


FIGURE 5. Diagrammatic depiction of the absorption of polygammaglutamated folates from food and the structure of polygammaglutamated folates and arrows pointing to the site of hydrolytic action of the novel brush border membrane bound folate hydrolase.

Either an increase in intracellular folate hydrolase or a decrease in folate polygammaglutamyl synthetase activity or both are associated with methotrexate resistance. This resistance to methotrexate occurs because methotrexate needs to be polygammaglutamated to be retained by cells to be active, just like folate. The ratio of expression of PSM and PSM' would be important for methotrexate retention. The flip side of this is that in cancer there is increased expression of PSM folate hydrolase outside of the cell. Thus, the enzymatic por-

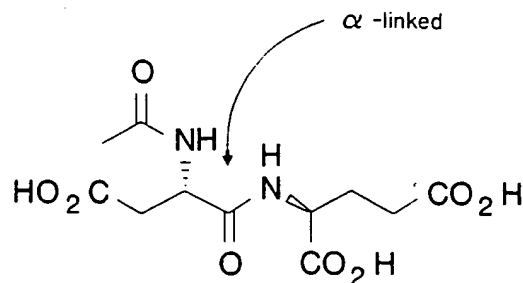
tion of PSM antigen is outside the cell and in a position that it could act on prodrugs, that is, polyglutamated therapeutic agents that otherwise cannot be transported into the cell. PSM antigen would remove the polyglutamates that would allow the drug to be transported into the cell. Consideration of these activities of PSM antigen should be useful in designing therapeutic strategies for metastatic prostatic cancer.

Adding to the uniqueness of the enzymatic function of PSM antigen, Carter and coworkers discov-

ered that a neurocarboxypeptidase enzyme they isolated from the rat brain had an 86% homology with bases 1106 to 2534 of the coding region of the PSM gene.¹⁵ The substrate for their neurocarboxypeptidase was *N*-acetylaspartylglutamic acid (NAAG), and they called the rat enzyme NAA-LADase, for *N*-acetylated alpha-linked acidic dipeptidase. These investigators found that LNCaP cells strongly expressed NAA-LADase activity.¹⁵ In the brain, *N*-acetylaspartylglutamate is a potential neurotransmitter substance that may act at glutamate excitatory amino acid receptors either directly or following the hydrolysis of NAAG with the concomitant release of glutamic acid (Fig. 6).

It is unclear that the neuropeptide NAAG exists in the prostate. Further, Pinto and coworkers found that the folate analogs had a much higher affinity for PSM antigen and thus are likely the preferred substrate. On the other hand, glutamate is found in high concentrations in the seminal plasma. However, glutamate would not be considered to have a neural-type role in the prostate as glutamate receptors are restricted to, and most strongly expressed in, the central nervous system (CNS). In the CNS, the blood-brain barrier serves to keep the concentration of glutamate relatively low. Interestingly, it was observed that the prostate expresses glutamate ion channel receptors in the stroma and basal cells.¹⁶ Indeed, there may be additional glutamate receptors present, as only antiGLU 2/3 and antiGLU 4 antibodies were used in the initial report.¹⁶ However, a cautionary view toward prostatic glutamate receptors is required because the results of the detection of glutamate receptors has been accomplished only by immunohistochemistry with polyclonal antibodies. Confirmation by other methods is required.¹⁶ The presence of glutamate receptors and an enzyme involved in dipeptidase activity relevant for glutamate-receptor function may be more than a coincidence, but it remains to be proven that such is the case.

It should be noted that the prostate exhibits many neuronal characteristics in its expressed proteins and receptors. In the prostate, there is a cell subset population with neuroendocrine features that makes a number of neuropeptide hormones. Their function is not clear. Neuroendocrine cells may represent a pathway from stem cell to differentiated cell and may be differentiated end cells. In neuroendocrine tumors, the secretion into the media of neuropeptides, such as neurotensin, endothelin, gastrin, and angiotensin, can result in an autocrine-growth signal. Some neuroendocrine cells attenuate autocrine-signal activation by the action of cell-surface peptidases. One such cell-surface peptidase with broad ability to inactivate neuroendocrine peptides is neutral endopeptidase



NAAG 1

N-acetylaspartyl-L-glutamate

FIGURE 6: Structural representation of the neuropeptide *N*-acetylaspartylglutamate (NAAG) with the arrow demonstrating the site of hydrolysis by rat brain NAA-LADase or by PSM antigen.

(NEP). NEP has been found to be absent in hormone-independent tumors, such as PC-3, and such cells have been found to be stimulated by neuropeptides.¹⁷ In LNCaP cells, androgens were found to upregulate the expression of this enzyme, while the absence of androgens was found to decrease the amount of NEP on the cell surface. Thus for NEP, it is easy to see how loss of its function could result in tumor progression to an androgen-independent phenotype.¹⁷ The activity of PSM antigen as a regulator of neuroendocrine function and growth of these cells is more difficult to discern. If the result of cleavage by cell-surface proteases active on the surface yields a carboxy-terminal acidic amino acid, then PSM would serve to activate or inactivate such a signal, depending on the activity of the carboxyl (-1) peptide. Investigations are currently underway to determine if there are possible neuroendocrine or other peptide/protein substrates for PSM antigen.

CONCLUSIONS

PSM antigen appears to be a potential diagnostic and therapeutic target for a number of reasons. Its expression is upregulated in prostate cancer and it has a fairly restricted expression, being found primarily in the prostate and proximal small intestine. It is expressed strongly in primary prostatic tumors and in the majority of disease metastatic to the lymph nodes or to the bone. The original antibody has had success as an imaging agent for locating the site of tumor, in patients with rising PSA values following radical prostatectomy, and is currently approved for these uses. However, as an imaging agent, it recognizes an intracellular region of the PSM molecule. The development of second-generation antibodies should be better able to bind

to the extracellular fraction of the molecule and should be more useful for imaging or therapeutic targeting. Such second-generation antibodies are already being developed, and initial reports have appeared in the literature.¹⁸

PSM antigen has been found to have activity as a unique membrane folate gamma-glutamyl hydrolase. In the normal prostate, PSM⁺ appears to predominate and is likely to be a cytosolic protein. This may cause the prostate to be an organ at risk for the development of folate-deficient states and to the induction of cancer. In cancer, PSM predominates, and as the enzyme is outside the cell, may provide an enzymatic activity that can be used to activate prodrugs that could be toxic to metastatic prostate tumor cells. PSM antigen is a unique hydrolase in that it also attacks alpha-linked glutamates with activity on the proposed neurotransmitter *N*-acetylaspartylglutamate. In the CNS, glutamate is considered to be a major neurotransmitter, with multiple forms of ionic and metabotropic glutamate receptors having been identified. In the prostate, there are neuroendocrine, secretory, and smooth muscle cells; their function with respect to glutamate, polyglutamated folate, or neuropeptides, and PSM antigen is currently unknown and requires further investigation.

Because of the tissue-specific, high-level expression of PSM, it may be possible to use the PSM-promoter region as part of a genetic vector for gene-specific activation of antiprostatic cancer therapies. An additional way to achieve specificity would be via immune activation. Peptides of PSM antigen have been observed to activate cytotoxic T-cell recognition.¹⁹ PSM antigen linked to an immunoadjuvant could also potentially serve to induce antiprostatic immunity in a fashion similar to those studies currently underway for PSA. As an immune target, PSM could either be used by itself or as part of a therapeutic vaccine cocktail. Thus, there are a number of potential uses of PSM as a diagnostic and therapeutic target in patients with prostatic cancer.

QUESTION AND ANSWER SECTION

Dr. William R. Fair: Mike, do you have any comments on probasin promoters or anything like that?

Dr. L. Michael Glode: No. The main question I have is whether you have had a chance to look at the restriction of activity in the transgenic model, in terms of using the promoter with some reporter gene?

Dr. Warren D.W. Heston: You mean in animals? We are presently undertaking that. We have not done that because it has not been totally clear ex-

actly where the start site is on the PSM promoter. We have some activity here that has been encouraging, but there have been a couple of little glitches that we want to make sure are totally ironed out.

Dr. Glode: It would be very intriguing to see if you could do the same thing that has been done with probasin in terms of the transgenic line. They have developed prostate cancer that looks really very interesting.

Dr. Heston: Exactly.

Dr. Glode: Of course, you might get duodenal tumors or brain tumors.

Dr. Heston: That is true. We do not know. That is exactly where transgenic animal models have been very useful. We are heading in that direction.

ACKNOWLEDGMENTS. A large thanks is extended to the many collaborators, colleagues, and fellows who have helped in the investigations of PSM antigen. These investigators include W.R. Fair, Sai Su, Tom Powell, Carlos Cordon-Cardo, John Pinto, William Tong, Polly Gregor, Ron Israeli, Stu Diamond, David Silver, Louis LaCombe, Bob Huryk, Liz Edwards, Ying Luo, Florian May, Joseph R. Bertino, Carrie Rinker-Schaeffer, and John Isaacs. In addition, thanks are given to Deanna James and Lew Freedman.

REFERENCES

1. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, and Murphy GP: LNCaP model of human prostatic carcinoma. *Cancer Res* 43: 1809-1818, 1991.
2. Horoszewicz JS, Kawinski E, and Murphy JP: Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. *Anticancer Res* 7: 927-935, 1987.
3. Israeli R, Powell CT, Fair W, and Heston WDW: Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res* 53: 227-230, 1993.
4. Su S, Fair W, Powell CT, and Heston WDW: Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. *Cancer Res* 55: 1441-1443, 1995.
5. Troyer J, Fang Q, Beckett ML, and Wright GL Jr: Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate specific membrane antigen. *Urol Oncol* 1: 29-37, 1995.
6. Israeli RS, Powell CT, Corr JG, Fair WR, and Heston WDW: Expression of the prostate-specific membrane antigen. *Cancer Res* 54: 1807-1811, 1994.
7. Wright GL Jr, Grob BM, Haley C, Grossman K, Newhall K, Petrylak D, Troyer J, Konchuba A, Schellhammer PF, and Moriarity R: Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology* 48: 326-334, 1996.
8. Troyer JK, Beckett ML, and Wright GL Jr: Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int J Cancer* 62: 552-558, 1995.
9. Silver DA, Pellicer I, Fair WR, Heston WDW, and Cordon-Cardo C: Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin Cancer Res* 3: 81-85, 1997.
10. Chandler CJ, Wang TTY, and Halstead CH: Pteroyl-polyglutamate hydrolase from human jejunal brush borders.

Purification and characterization. *J Biol Chem* 261: 928-933, 1986.

11. Pinto JT, Suffoletto BP, Berzin TM, Qiao CH, Lin S, Tong WP, May FM, Mukherjee B, and Heston WDW: Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin Cancer Res* 2: 1445-1451, 1996.

12. Jennings E: Folic acid as a cancer preventing agent. *Med Hypotheses* 45: 297-303, 1995.

13. Heston WDW: Prostatic polyamines and polyamine targeting as a new approach to the therapy of prostatic cancer. *Cancer Surv* 11: 217-238, 1991.

14. Jarrard DF, Bova GS, and Isaacs WB: DNA methylation, molecular genetic, and linkage studies in prostate cancer. *Prostate* 6(suppl): 36-44, 1996.

15. Carter RE, Feldman AR, and Coyle JT: Prostate-specific membrane antigen is a hydrolase with substrate and phar-

macologic characteristics of a neuropeptidase. *Proc Natl Acad Sci USA* 93: 749-753, 1996.

16. Heston WDW, Silver DA, Pellicer I, Fair WR, and Cordon-Cardo C: Ionotropic glutamate receptor distribution in prostate tissue (abst 809). *J Urol* 155:513A, 1996.

17. Papandreou P, Freeman R, Xu Y, Scheinberg D, Maslak P, and Scher HI: Neutral endopeptidase regulation by androgen in prostate cancer cells (abst). *Proc Amer Assoc Cancer Res* 37: 244, 1996.

18. Murphy GP, Tino WT, Holmes EH, Boynton AL, Erickson SJ, Bowes VA, Barren RJ, Tjoa BA, Misrock SL, Ragde H, and Kenny GM: Measurement of prostate-specific membrane antigen in the serum with a new antibody. *Prostate* 28: 266-271, 1996.

19. Tjoa B, Boynton A, Kenny G, Ragde H, Misrock SL, and Murphy GP: Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. *Prostate* 28: 65-69, 1996.

Fundamental Immunology

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be expressed on macrophages, monocytes, and granulocytes, where their importance in adherence, enhanced phagocytosis, and chemotactic activity is well known. Suci: receptors might permit B cells to interact with other cells, but a clear role has not been determined. Complement receptors are also detected by rosetting techniques.

Another important class of receptors are the **cytokine receptors**. Cytokines (the general classification) are secretory products of particular cells that act on other cells. Lymphokines refer to products of lymphocytes, while monokines are produced and released by monocytes. These secretions, in turn, act on other cell populations. For example, the cytokine, interleukin 1 (IL-1), acts on lymphocytes, NK cells, and fibroblasts. The roles of several important lymphokines and monokines in immune responses are discussed in Chapter 12, Induction and Regulation of Immune Responses. **B cells** possess receptors for IL-1, a cytokine produced by several cell types, and **B cell growth factor** (lymphokines produced by activated T cells, which include IL-2). B cells also possess receptors for factors produced by activated T cells, which contribute to B cell differentiation. T cells have receptors for IL-1 and IL-2.

Yet another class of receptors are the **mitogen receptors**. Substances that promote cell division, or mitosis, are called **mitogens**. Thus, the ligands that bind to these receptors promote the proliferation of B or T cells. T cell mitogens differ from B cell mitogens. One class of mitogen consists of **lipopolysaccharides (LPS)** derived from the cell walls of gram-negative bacteria. These substances can act as specific antigens in mice at one concentration and as a mitogen, or B cell polyclonal activators, at higher concentrations. Thus, in the B cell repertoire of antigen-reactive clones, a few can react at low LPS concentrations, while any clone can react at higher LPS concentrations. *Human B cells do not exhibit LPS receptors*, but they can be stimulated by pokeweed (*Phytolacca americana*) mitogens.

A second class of mitogens is the **lectins**. These are glycoproteins produced by some plants that bind specifically to certain sugar residues present in **glycoprotein receptors** exhibited on certain cell surfaces. *Phytohemagglutinin (PHA)* is a lectin from the red kidney bean (*Phaseolus vulgaris*) that binds to both T and B lymphocytes, but it only stimulates T cells to divide. *Con-*

canavalin A (Con A), a lectin extracted from the black bean (*Conavalia ensiformis*), binds to T cells and acts as a T cell mitogen for both immature and mature cells. Selective T cell mitogens have served to distinguish T cells from B cells and act as **polyclonal T cell activators**. An antigen stimulates a specific antigen-reactive clone of T or B cells. T cell mitogens, on the other hand, stimulate any clone of T cells, regardless of their binding specificity for antigen. In this regard, mitogens have been very useful in the study of T cell function.

Receptors also are present for other signal molecules, for example, hormones and neurotransmitters. Receptors for endorphins and acetylcholine have been identified on lymphocytes. Although their precise role is not yet clear, they undoubtedly mediate communications from the other regulatory systems. In addition, T cells express receptors for nonspecific binding to erythrocytes. This reaction is energy dependent since it can be blocked by metabolic poisons; moreover, it is a nonantigenic interaction.

Most membrane antigenic determinants and receptors will undergo **surface redistribution** when they combine with complementary molecules at body temperature. After several minutes, the material aggregates into patches over the entire cell surface. Dispersed patches then coalesce and localize at one pole of the cell, forming a **polar cap** (figure 4.12). The process may be easily visualized by staining cells with fluorescent antibodies directed to the Ig receptors. If monovalent labeled antibodies are used (rather than divalent or multivalent), redistribution doesn't occur, suggesting that **crosslinking of receptors** is necessary for patching and capping. This capping event, which is usually followed by internalization of the components (pinocytosis) by means of membrane vesicles, requires metabolic activity. Therefore, conditions that depress mobility of the fluid membrane inhibit or block capping (e.g., low temperatures). Cells normally lose surface determinants and/or receptors. Accordingly, after B cells lose sIg, they can **reexpress these receptors** within 8 hours, suggesting dynamic turnover in surface markers. The capping and internalization events suggest that this process might be a key step in cell activation, not only for B and T lymphocytes, but for other cells of the immune system as well. The intracellular events that follow are now the focus of research interest.

Expression of the Prostate-specific Membrane Antigen¹

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ABSTRACT

We have recently cloned a 2.65-kilobase complementary DNA (cDNA) encoding the prostate-specific membrane antigen (PSM) recognized by the 7E11-C5.3 anti-prostate monoclonal antibody. Immunohistochemical analysis of the LNCaP, DU-145, and PC-3 prostate cancer cell lines for PSM expression using the 7E11-C5.3 antibody reveals intense staining in the LNCaP cells with no detectable expression in both the DU-145 and PC-3 cells. Coupled *in vitro* transcription/translation of the 2.65-kilobase full-length PSM cDNA yields an *M_r* 84,000 protein corresponding to the predicted polypeptide molecular weight of PSM. Posttranslational modification of this protein with pancreatic canine microsomes yields the expected *M_r* 100,000 PSM antigen. Following transfection of PC-3 cells with the full-length PSM cDNA in a eukaryotic expression vector, we detect expression of the PSM glycoprotein by Western analysis using the 7E11-C5.3 monoclonal antibody. Ribonuclease protection analysis demonstrates that the expression of PSM mRNA is almost entirely prostate specific in human tissues. PSM expression appears to be highest in hormone-deprived states and is hormonally modulated by steroids, with 5- α -dihydrotestosterone down-regulating PSM expression in the human prostate cancer cell line LNCaP by 8–10-fold, testosterone down-regulating PSM by 3–4-fold, and corticosteroids showing no significant effect. Normal and malignant prostatic tissues consistently show high PSM expression, whereas we have noted heterogeneous, and at times absent, expression of PSM in benign prostatic hyperplasia. LNCaP tumors implanted and grown both orthotopically and *s.c.* in nude mice abundantly express PSM, providing an excellent *in vivo* model system to study the regulation and modulation of PSM expression.

INTRODUCTION

Prostate cancer is among the most significant medical problems in the United States because the disease is now the most common malignancy diagnosed in American males. In 1992, there were over 132,000 new cases of prostate cancer detected with over 36,000 deaths attributable to the disease, representing a 17.3% increase over 4 years (1). Five-year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. The rapid increase in the number of cases appears to result in part from an increase in disease awareness as well as the widespread use of clinical markers such as the secreted proteins PSA⁴ and PAP (2). We have recently reported the molecular cloning, from the human prostatic adenocarcinoma cell line LNCaP, of a complementary DNA encoding a *M_r* 100,000 prostate-specific membrane glycoprotein (3), recognized by the anti-prostate monoclonal antibody 7E11-C5.3 (4). The LNCaP cell line was derived from a supraclavicular lymph node of a patient with hormone-refractory prostate cancer (5). Currently, LNCaP cells provide the best *in vitro* model system to study human prostate cancer since they produce all three prostatic

biomarkers, PSA, PAP, and PSM. The cells possess an aneuploid male karyotype with a Y chromosome, express a high affinity androgen receptor, and are hormonally responsive to both testosterone and DHT. Because PSM appears to be a transmembrane glycoprotein, it is considered an attractive target for both antibody-directed imaging and targeting of prostatic tumor deposits (6). In this paper, we demonstrate expression of PSM protein in LNCaP cell membranes and in PC-3 cells transfected with PSM cDNA. In addition, we report the characterization of PSM mRNA expression in human tissues and in response to steroid hormones.

MATERIALS AND METHODS

Cells and Reagents. The LNCaP, DU-145, and PC-3 cell lines were obtained from the American Type Culture Collection. Details regarding the establishment and characteristics of these cell lines have been published previously (5, 7, 8). Unless specified otherwise, LNCaP cells were grown in RPMI 1640 supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD) in a CO₂ incubator at 37°C. DU-145 and PC-3 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. All cell media were obtained from the MSKCC media preparation facility. Restriction and modifying enzymes were purchased from Gibco-BRL unless otherwise specified. The modified 7E11-C5.3 anti-PSM monoclonal antibody CYT-356 was provided by the Cytogen Corporation, Princeton, NJ. All other chemical reagents were of the highest grade possible and were obtained from Sigma Chemical Company, St. Louis, MO.

Immunohistochemical Detection of PSM. We used the avidin-biotin method of detection to analyze prostate cancer cell lines for PSM antigen expression (9). Cell cytopins were made on glass slides using 5 × 10⁴ cells/100 μ l per slide. Slides were washed twice with phosphate-buffered saline and then incubated with the appropriate blocking serum for 20 min. The blocking serum was drained off, and the cells were incubated with diluted 7E11-C5.3 (5 μ g/ml) monoclonal antibody for 1 h. Samples were then washed with phosphate-buffered saline and sequentially incubated with secondary antibodies for 30 min and with avidin-biotin complexes for 30 min. Diaminobenzidine served as our chromogen and color development followed by hematoxylin counterstaining and mounting. Duplicate cell cytopins were used as controls for each experiment. As a positive control, the anti-cytokeratin monoclonal antibody CAM 5.2 was used following the same procedure described above. Human EJ bladder carcinoma cells served as a negative control.

***In Vitro* Transcription/Translation of PSM Antigen.** Plasmid 55A containing the full-length 2.65-kilobase PSM cDNA in the plasmid pSPORT 1 (Gibco-BRL) was transcribed *in vitro* using the Promega TNT system (Promega Corp., Madison, WI). T7 RNA polymerase was added to the cDNA in a reaction mixture containing rabbit reticulocyte lysate, an amino acid mixture lacking methionine, buffer, and [³⁵S]methionine (Amersham, Arlington Heights, IL), and incubated at 30°C for 90 min. Posttranslational modification of the resulting protein was accomplished by the addition of pancreatic canine microsomes into the reaction mixture (Promega Corp.). Protein products were analyzed by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels which were subsequently treated with Amplify autoradiography enhancer (Amersham) according to the manufacturers instructions and dried at 80°C in a vacuum dryer. Gels were autoradiographed overnight at -70°C using Hyperfilm MP (Amersham).

Transfection of PSM into PC-3 Cells. The full-length PSM cDNA was subcloned into the pREP7 eukaryotic expression vector (Invitrogen, San Diego, CA). Plasmid DNA was purified from transformed DH5- α bacteria (Gibco-BRL) using Qiagen maxi-prep plasmid isolation columns (Qiagen, Inc., Chatsworth, CA). Purified plasmid DNA (6–10 μ g) was diluted with 900 μ l of Opti-mem media (Gibco-BRL) and mixed with 30 μ l of Lipofectin

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⁴ The abbreviations used are: PSA, prostate-specific antigen; PAP, prostatic acid phosphatase; DHT, 5- α -dihydrotestosterone; PSM, prostate-specific membrane antigen; cDNA, complementary DNA; TS, Tris-HCl; TS-X, Triton-X 100; MSKCC, Memorial Sloan-Kettering Cancer Center.

reagent (Gibco-BRL) which had been previously diluted with 900 μ l of Optimum media. This mixture was added to T-75 flasks of 40–50% confluent PC-3 cells in Optimum media. After 24–36 h, cells were trypsinized and split into 100-mm dishes containing RPMI 1640 supplemented with 10% fetal calf serum and 1 mg/ml of Hygromycin B (Calbiochem, La Jolla, CA). The dose of Hygromycin B used was determined previously by a time course/dose response cytotoxicity assay. Cells were maintained in this media for 2–3 weeks with changes of media and Hygromycin B every 4–5 days until discrete colonies appeared. Colonies were isolated using 6-mm cloning cylinders and expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 plasmid alone. RNA was isolated from the transfected cells, and PSM mRNA expression was detected by both RNase protection analysis (described later) and by Northern analysis.

Western Blot Detection of PSM Expression. Crude protein lysates were isolated from LNCaP, PC-3, and PSM-transfected PC-3 cells as described previously (10). LNCaP cell membranes were also isolated according to published methods (10). Protein concentrations were quantitated by the Bradford method using the Bio-Rad protein reagent kit (Bio-Rad, Richmond, CA). Following denaturation, 20 μ g of protein was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel at 25 mA for 4 h. Gels were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA) overnight at 4°C. Membranes were blocked in 0.15 M NaCl-0.01 M TS plus 5% bovine serum albumin followed by a 1-h incubation with 7E11-C5.3 monoclonal antibody (10 μ g/ml). Blots were washed 4 times with 0.15 M NaCl-0.01 M TS-0.05% TS-X and incubated for 1 h with rabbit anti-mouse IgG (Accurate Scientific, Westbury, NY) at a concentration of 10 μ g/ml. Blots were then washed 4 times with TS-X and labeled with 125 I-Protein A (Amersham) at a concentration of 1 million cpm/ml. Blots were then washed 4 times with TS-X and dried on Whatman 3MM paper, followed by overnight autoradiography at -70°C using Hyperfilm MP (Amersham).

Orthotopic and s.c. LNCaP Tumor Growth in Nude Mice. LNCaP cells were harvested from subconfluent cultures by a 1-min exposure to a solution of 0.25% trypsin and 0.02% EDTA. Cells were resuspended in RPMI 1640 with 5% fetal bovine serum and washed and diluted in either Matrigel (Collaborative Biomedical Products, Bedford, MA) or calcium and magnesium-free Hank's balanced salt solution. Only single cell suspensions with greater than 90% viability by trypan blue exclusion were used for *in vivo* injection. Male athymic Swiss (*nu/nu*) nude mice 4–6 weeks of age were obtained from the MSKCC animal facility. For s.c. tumor cell injection, one million LNCaP cells resuspended in 0.2 ml of Matrigel were injected into the hindlimb of each mouse using a disposable syringe fitted with a 28-gauge needle. For orthotopic injection, mice were first anesthetized with an i.p. injection of pentobarbital and placed in the supine position. The abdomen was cleansed with Betadine, and the prostate was exposed through a midline incision. LNCaP tumor cells (2.5 million) in 0.1 ml were injected directly into either ventral lobe using a 1-ml disposable syringe and a 28-gauge needle. LNCaP cells with and without Matrigel were injected. Abdominal closure was achieved in one layer using Autoclip wound clips (Clay Adams, Parsippany, NJ). Tumors were harvested in 6–8 weeks, confirmed histologically by faculty of the MSKCC Pathology Department, and frozen in liquid nitrogen for subsequent RNA isolation.

RNA Isolation. Total cellular RNA was isolated from cells and tissues by standard techniques (11, 12) as well as by using RNazol B (Cinna/Biotech, Houston, TX). RNA concentrations and quality were assessed by UV spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis. Human tissue total RNA samples were purchased from Clontech Laboratories, Inc., Palo Alto, CA.

Ribonuclease Protection Assays. A portion of the PSM cDNA was subcloned into the plasmid vector pSPORT 1 (Gibco-BRL), and the orientation of the cDNA insert relative to the flanking T7 and SP6 RNA polymerase promoters was verified by restriction analysis. Linearization of this plasmid upstream of the PSM insert followed by transcription with SP6 RNA polymerase yields a 400-nucleotide antisense RNA probe, of which 350 nucleotides should be protected from RNase digestion by PSM RNA. This probe was used in Fig. 4. Plasmid IN-20 containing a 1-kilobase partial PSM cDNA in the plasmid pCR II (Invitrogen) was also used for riboprobe synthesis. IN-20 linearized with *Xmn*I (Gibco-BRL) yields a 298 nucleotide antisense RNA probe when transcribed using SP6 RNA polymerase, of which 260 nucleotides should be protected from RNase digestion by PSM mRNA. This probe was used in Figs. 5 and 6. Probes were synthesized using SP6 RNA polymerase

(Gibco-BRL), rNTPs (Gibco-BRL), RNasin (Promega), and [32 P]CTP (NEN, Wilmington, DE) according to published protocols (13). Probes were purified over NENSORB 20 purification columns (NEN), and approximately 1 million cpm of purified, radiolabeled PSM probe was mixed with 10 μ g of each RNA and hybridized overnight at 45°C using buffers and reagents from the RPA II kit (Ambion, Austin, TX). Samples were processed as per manufacturer's instructions and analyzed on 5% polyacrylamide/7 M urea denaturing gels using Seq ACRYL reagents (ISS, Natick, MA.). Gels were preheated to 55°C and run for approximately 1–2 h at 25 watts. Gels were then fixed for 30 min in 10% methanol-10% acetic acid, dried onto Whatman 3MM paper at 80°C in a Bio-Rad vacuum dryer, and autoradiographed overnight with Hyperfilm MP (Amersham). Quantitation of PSM expression was determined by using a scanning laser densitometer (LKB, Piscataway, NJ).

Steroid Modulation Experiment. LNCaP cells (2 million) were plated onto T-75 flasks in RPMI 1640 supplemented with 5% fetal calf serum and grown 24 h until approximately 30–40% confluent. Flasks were then washed several times with phosphate-buffered saline, and RPMI supplemented with 5% charcoal-extracted serum was added. Cells were then grown for another 24 h, at which time dihydrotestosterone, testosterone, estradiol, progesterone, and dexamethasone (Sieraloids, Inc., Wilton, NH) were added at a final concentration of 2 nM. Cells were grown for another 24 h, and RNA was then harvested as described previously; PSM expression was analyzed by ribonuclease protection analysis.

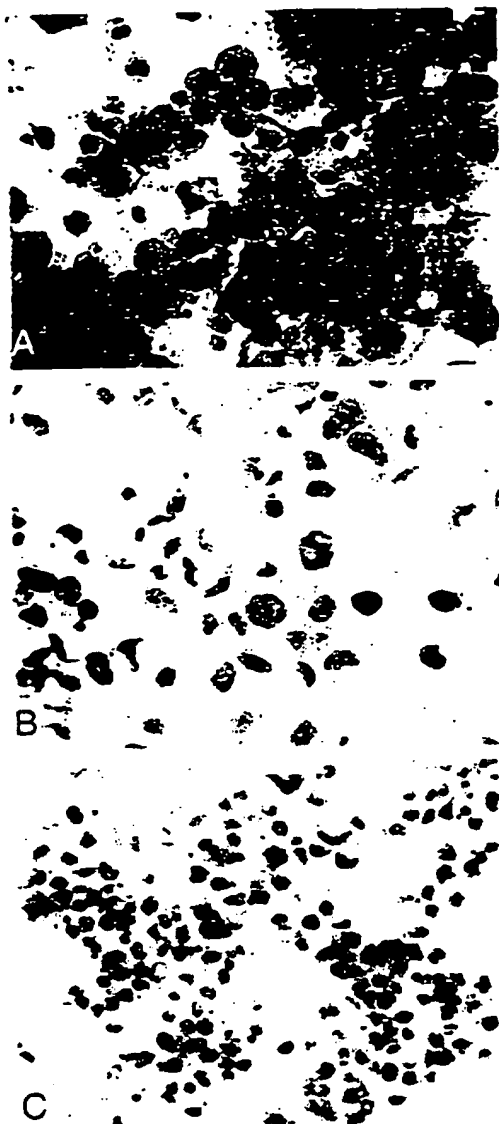
RESULTS

Immunohistochemical Detection of PSM. Using the 7E11-C5.3 anti-PSM monoclonal antibody, PSM expression is clearly detectable in the LNCaP prostate cancer cell line but not in the PC-3 and DU-145 cell lines (Fig. 1) in agreement with results published previously (4).

***In Vitro* Transcription/Translation of PSM Antigen.** As shown in Fig. 2, coupled *in vitro* transcription/translation of the 2.65-kilobase full-length PSM cDNA yields a *M*, 75,000–80,000 protein species in agreement with the expected protein product from the 750-amino acid PSM open reading frame. We have not investigated the reason for the two bands seen in the first lane. This may represent proteolytic degradation. Following posttranslational modification using pancreatic canine microsomes, the major band observed is a *M*, 100,000–110,000 glycosylated protein species consistent with the mature, native PSM antigen.

Detection of PSM Antigen in LNCaP Cell Membranes and Transfected PC-3 Cells. PC-3 cells transfected with the full-length PSM cDNA in the pREP7 expression vector were assayed for expression of PSM mRNA by Northern analysis (data not shown). A clone with high PSM mRNA expression was selected for PSM antigen analysis by Western blotting using the 7E11-C5.3 antibody. In Fig. 3, the *M*, 100,000 PSM antigen is well expressed in LNCaP membrane fractions and crude cell lysate (Fig. 3, *Lanes 1* and 2) as well as in PSM-transfected PC-3 cells (Fig. 3, *Lane 4*) but not in native PC-3 cells (Fig. 3, *Lane 3*). This detectable expression in the transfected PC-3 cells (Fig. 3, *Lane 4*) proves that the previously cloned 2.65-kilobase PSM cDNA encodes the antigen recognized by the 7E11-C5.3 anti-prostate monoclonal antibody and that the antigen is being appropriately glycosylated in the PC-3 cells since the antibody recognizes a carbohydrate-containing epitope on PSM.

PSM mRNA Expression. Expression of PSM mRNA in normal human tissues was analyzed using ribonuclease protection assays. Tissue expression of PSM appears predominantly within the prostate, with very low levels of expression detectable in human brain and salivary gland (Fig. 4). We have also noted on occasion detectable PSM expression in normal human small intestine tissue; however, this mRNA expression is variable depending upon the specific riboprobe used (data not shown). All samples of normal human prostate and human prostatic adenocarcinoma assayed (*n* = 18) have revealed clearly detectable PSM expression, whereas we have noted generally decreased or absent expression of PSM in tissues exhibiting benign



Immunohistochemical detection of PSM antigen expression in prostate cell lines. *A*, LNCaP cells; *B*, DU-145 cells; *C*, PC-3 cells, respectively (both negative).

in human LNCaP tumors grown both subcutaneously and s.c. in nude mice, we detected abundant PSM mRNA with or without the use of Matrigel, which is required for s.c. implanted LNCaP cells (Fig. 5). Since PSA has been shown to be up-regulated by androgens (14), we investigated the androgen responsiveness of PSM which has been noted to be down-regulated following hormone deprivation. PSM mRNA expression is distinctly modulated by the presence of steroids in clinical doses (Fig. 6). DHT down-regulated expression by 75% after 24 h, and testosterone diminished PSM expression by 50%. Estradiol and progesterone also down-regulated PSM expression in LNCaP cells, perhaps as a result of binding to the mutated androgen receptor known to exist in the LNCaP cell. Overall, PSM mRNA is highest in the untreated LNCaP cells grown in steroid-media, a situation that we propose simulates the hormone-deprived (castrate) state *in vivo*. This experiment was repeated at dosages ranging from 2–200 nM and at time points from 6 h to

7 days with similar results; maximal down-regulation of PSM mRNA was seen with DHT at 24 h at doses of 2–20 nM (data not shown). A separate RNase protection assay was performed using a human acidic ribosomal phosphoprotein PO probe (15) in the same reaction as the PSM probe. It was noted that the expression of the PO mRNA was not affected by steroid treatment, whereas the changes in PSM expression were identical to those in Fig. 6 (data not shown).

DISCUSSION

In order to better understand the biology of the human prostate in both normal and neoplastic states, we need to enhance our knowledge

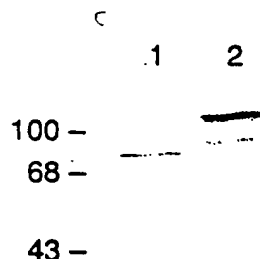


Fig. 2. Autoradiogram of protein gel revealing products of PSM coupled *in vitro* transcription/translation. Nonglycosylated PSM polypeptide is seen at approximately M_r 75,000–80,000 (Lane 1), and PSM glycoprotein synthesized following the addition of microsomes is seen at M_r 100,000–110,000 (Lane 2).

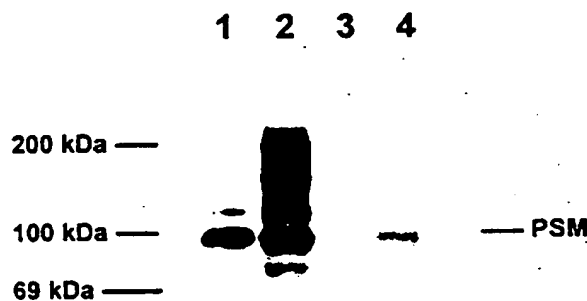


Fig. 3. Western blot analysis detecting PSM expression in transfected non-PSM expressing PC-3 cells. M_r 100,000 PSM glycoprotein species is clearly seen in LNCaP membranes (Lane 1), LNCaP crude lysate (Lane 2), and PSM-transfected PC-3 cells (Lane 4) but is undetectable in native PC-3 cells (Lane 3).

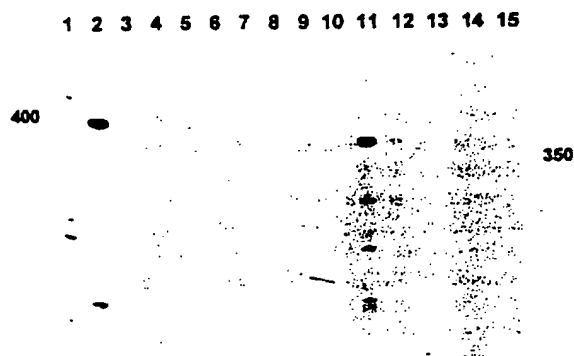


Fig. 4. Autoradiogram of ribonuclease protection gel assaying for PSM mRNA expression in normal human tissues. Lane 1, radiolabeled 1-kilobase DNA ladder (Gibco-BRL). Lane 2, undigested probe is 400 nucleotides and expected protected PSM band is 350 nucleotides. Lane 3, tRNA control. A strong signal is seen in human prostate (Lane 11), with very faint but detectable signals seen in human brain (Lane 4) and human salivary gland (Lane 12). Other samples include Lane 5, kidney; Lane 6, liver; Lane 7, lung; Lane 8, mammary gland; Lane 9, pancreas; Lane 10, placenta; Lane 12, salivary gland; Lane 13, skeletal muscle; Lane 14, spleen; and Lane 15, testis.

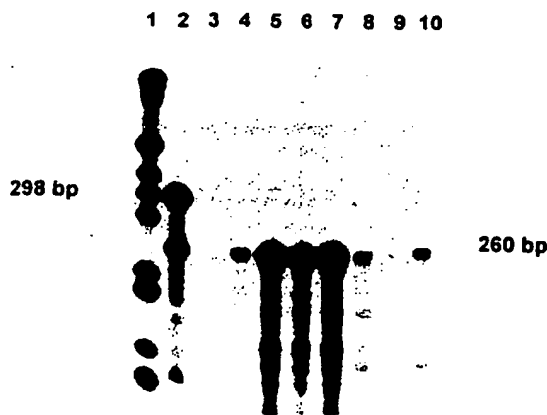


Fig. 5. Autoradiogram of ribonuclease protection gel assaying for PSM mRNA expression in LNCaP tumors grown in nude mice and in human prostatic tissues. Lane 1, 32 P-labeled 1-kilobase DNA ladder. Lane 2, 298-nucleotide undigested probe. Lane 3, tRNA control. A 260–270 nucleotide band in the undigested probe lane (Lane 2) is seen and probably represents a partial RNA transcript. This RNA is completely degraded by RNase in the absence of PSM mRNA as seen in the tRNA control (Lane 3). PSM mRNA expression is clearly detectable in LNCaP cells (Lane 4), orthotopically grown LNCaP tumors in nude mice with and without Matrigel (Lanes 5 and 6), and s.c. implanted and grown LNCaP tumors in nude mice (Lane 7). PSM mRNA expression is also seen in normal human prostate (Lane 8) and in a moderately differentiated human prostatic adenocarcinoma (Lane 10). Very faint expression is seen in a sample of human prostatic tissue with benign hyperplasia (Lane 9).

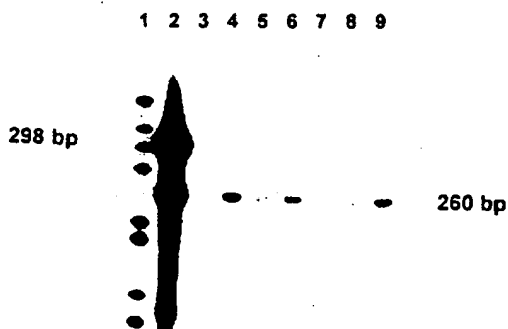


Fig. 6. Ribonuclease protection assay for PSM expression in LNCaP cells treated with physiological doses of various steroids for 24 h. Lane 1, 32 P-labeled DNA ladder. Lane 2, 298-nucleotide undigested probe. Lane 3, tRNA control. PSM mRNA expression is highest in untreated LNCaP cells in charcoal-stripped media (Lane 4). We see significantly diminished PSM expression in LNCaP cells treated with DHT (Lane 5), testosterone (Lane 6), estradiol (Lane 7), and progesterone (Lane 8), with little response to dexamethasone (Lane 9).

by studying the various proteins and other features that are unique to this important gland. Previous research has provided two valuable prostatic biomarkers, PAP and PSA, both of which have had a significant impact on the diagnosis, treatment, and management of prostate malignancies. Our present work describing the preliminary characterization of the PSM reveals it to be a gene with many interesting features. PSM is almost entirely prostate specific, as are PAP and PSA, and as such may enable further delineation of the unique functions and behavior of the prostate. The predicted sequence of the PSM protein (3) and its presence in the LNCaP cell membrane as determined by Western blotting and immunohistochemistry indicate that it is an integral membrane protein. Thus, PSM provides an attractive cell surface epitope for antibody-directed diagnostic imaging and cytotoxic targeting modalities.⁵ The ability to synthesize the

PSM antigen *in vitro* and to produce tumor xenografts maintaining high levels of PSM expression provides us with a convenient and attractive model system to further study and characterize the regulation and modulation of PSM expression. Also, the high level of PSM expression in the LNCaP cells provides an excellent *in vitro* model system. Since PSM expression is hormonally responsive to steroids and may be highly expressed in hormone-refractory disease (16), it is imperative to elucidate the potential role of PSM in the evolution of androgen-independent prostate cancer. The detection of PSM mRNA expression in minute quantities in brain, salivary gland, and small intestine warrants further investigation, although these tissues were negative for expression of PSM antigen by immunohistochemistry using the 7E11-C5.3 antibody (17). In all of these tissues, particularly small intestine, we detected mRNA expression using a probe corresponding to a region of the PSM cDNA near the 3' end, whereas we were unable to detect expression when using a 5' end PSM probe. These results may indicate that the PSM mRNA transcript undergoes alternative splicing in different tissues. Previous protein studies have suggested that the 7E11-C5.3 antibody may actually detect two other slightly larger protein species in addition to the M_r 100,000 PSM antigen (18). These other protein species can be seen in the LNCaP lysate and membrane samples in Fig. 3. Possible origins of these proteins include alternatively spliced PSM mRNA, other genes distinct from but closely related to PSM, or different posttranslational modifications of the PSM protein. We are currently investigating these possibilities.

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REFERENCES

- Coffey, D. S. Prostate cancer—an overview of an increasing dilemma. *Cancer* (Phila.), 71 (Suppl.): 880–886, 1993.
- Chiarodo, A. National Cancer Institute roundtable on prostate cancer: future research directions. *Cancer Res.*, 51: 2498–2505, 1991.
- Israeli, R. S., Powell, C. T., Fair, W. R., and Heston, W. D. W. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.*, 53: 227–230, 1993.
- Horoszewicz, J. S., Kawinski, E., and Murphy, G. P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. *Anticancer Res.*, 7: 927–936, 1987.
- Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. P. LNCaP model of human prostatic carcinoma. *Cancer Res.*, 43: 1809–1818, 1983.
- Abdel-Nabi, H., Wright, G. L., Gulfo, J. V., Petrylak, D. P., Neal, C. E., Texter, J. E., Begun, F. P., Tyson, I., Heal, A., Mitchell, E., Purnell, G., and Harwood, S. J. Monoclonal antibodies and radioimmunoconjugates in the diagnosis and treatment of prostate cancer. *Semin. Urol.*, 10: 45–54, 1992.
- Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., and Paulson, D. F. Isolation of a human prostate carcinoma cell line (DU-145). *Int. J. Cancer*, 21: 274–281, 1978.
- Kaign, M. E., Narayan, K. S., Ohnuki, Y., and Lechner, J. F. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest. Urol.*, 17: 16–23, 1979.
- Hsu, S. M., Raine, L., and Fanger, H. Review of present methods of immunohistochemical detection. *Am. J. Clin. Pathol.*, 75: 734–738, 1981.
- Harlow, E., and Lane, D. *Antibodies: A Laboratory Manual*, p. 449. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.
- Glisin, V., Crkvenjakov, R., and Byus, C. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry*, 13: 2633–2637, 1974.
- Aviv, H., and Leder, P. Purification of biologically active globin messenger RNA by chromatography on oligo-thymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA*, 69: 1408–1412, 1972.
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T. A., Zinn, K., and Green, M. R. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization

⁵ J. Gulfo, Cytogen Corporation (Princeton, NJ), personal communication.

- probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.*, 12: 7035-7056, 1984.
14. Henttu, P., Liao, S., and Vihko, P. Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. *Endocrinology*, 130: 766-772, 1992.
15. Laborda, J. 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. *Nucleic Acids Res.*, 19: 3998, 1991.
16. Axelrod, H. R., Gilman, S. C., D'Aleo, C. J., Petrylak, D., Reuter, V., Gulfo, J. V., Saad, A., Cordon-Cardo, C., and Scher, H. I. Preclinical results and human immunohistochemical studies with ⁹⁰Y-CYT-356: a new prostatic cancer therapeutic agent. *AUA Proceedings*, Abstract 596, 1992.
17. Lopes, A. D., Davis, W. L., Rosenstraus, M. J., Uveges, A. J., and Gilman, S. C. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from antiprostata monoclonal antibody 7E11-CS. *Cancer Res.*, 50: 6423-6429, 1990.
18. Troyer, J. K., Qi, F., Beckett, M. L., Murningstar, M. M., and Wright, G. L. Molecular characterization of the 7E11-CS prostate tumor-associated antigen. *AUA Proceedings*, Abstract 482, 1993.

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Molecular Cloning of a Complementary DNA Encoding a Prostate-specific Membrane Antigen¹

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Abstract

Recently, a novel *M*_r 100,000 prostate-specific membrane glycoprotein (PSM) has been detected by the prostate-specific monoclonal antibody 7E11-C5, raised against the human prostatic carcinoma cell line LNCaP. The PSM antigen is expressed exclusively by normal and neoplastic prostate cells and metastases. We now report the molecular cloning of a full-length 2.65-kilobase complementary DNA encoding the PSM antigen from a human LNCaP complementary DNA library by polymerase chain reaction using degenerate oligonucleotide primers. Analysis of the complementary DNA sequence has revealed that a portion of the coding region, from nucleotide 1250 to 1700, has 54% homology to the human transferrin receptor mRNA. The deduced polypeptide has a putative transmembrane domain enabling the delineation of intra- and extracellular portions of this antigen. In contrast to prostate-specific antigen and prostatic acid phosphatase which are secreted proteins, PSM as an integral membrane protein may prove to be effective as a target for imaging and cytotoxic targeting modalities.

Introduction

Prostate cancer represents the most common malignancy in American males and is the second leading cause of cancer-related death in the male population (1). The disease has diverse manifestations, from slow growing, indolent primary lesions to aggressive, refractory metastatic disease, with a predilection toward bone metastases. PAP³ was one of the earliest serum markers for detecting metastatic spread of prostate cancer (1); this marker has been augmented in recent years by PSA (1). PSA has been shown to correlate with tumor burden, serve as an indicator of metastatic involvement, and provide an excellent parameter for following the response to surgery, irradiation, and androgen ablation therapy in patients with prostate cancer. Both of these proteins are secreted and are readily measured in the serum, as well as in prostatic secretions. The LNCaP human prostate cancer cell line was established from a metastatic lymph node from a heavily pretreated patient with hormone-refractory prostate carcinoma (2). This cell line serves as the best *in vitro* model for human prostatic carcinoma in that it possesses an aneuploid male karyotype, maintains prostatic differentiation functionality in that it produces PAP and PSA, and expresses a high affinity androgen receptor. Cell membranes were isolated from these cells and mice were immunized with them to form hybridomas. A prostate-specific monoclonal antibody was generated using spleen cells of mice immunized with LNCaP cell membranes and designated 7E11-C5 (3). The antibody staining exhibited a mem-

brane location with LNCaP cells reacting strongly. Both benign and neoplastic prostate cells stained positively, with more intense staining seen with malignant cells. Lymph node and bone metastases also stain positively with the antibody, with the highest expression seen in hormone-refractory lesions (4). The epitope of the antibody has been shown to include a carbohydrate portion of the PSM antigen and the antigen has an apparent molecular weight of approximately 100,000 on SDS-polyacrylamide gel electrophoresis (5). In this paper, we report the molecular cloning of a full-length cDNA encoding the *M*_r 100,000 prostate-specific membrane antigen.

Materials and Methods

Cells and Reagents. The LNCaP, DU-145, and PC-3 cell lines used were obtained from the American Type Culture Collection. Details regarding the development of these cell lines and their characteristics have been published previously (2, 6, 7). Unless specified otherwise, LNCaP cells were grown in RPMI 1640 supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL) in a CO₂ incubator at 37°C. DU-145 and PC-3 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. All media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were obtained from Sigma Chemical Company, St. Louis, MO. The modified 7E11-C5 monoclonal antibody to the PSM antigen (CYT-356) was obtained from CytoGen Corporation, Princeton, NJ.

Immunoprecipitation of the PSM Antigen. LNCaP cells were starved in methionine-depleted RPMI for 2 h, after which [³⁵S]methionine was added at 100 µCi/ml and the cells were grown for another 16–18 h. Cells were then washed and lysed by addition of 1 ml of lysis buffer [1% Triton X-100, 50 mM Hepes (pH 7.5), 10% glycerol, 15 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 1 mM [ethylenebis(oxyethylenetriol)]tetraacetic acid] and incubated for 20 min at 4°C. Lysates were precleared by mixing with Pansorbin cells (Calbiochem) for 90 min at 4°C. Cell lysates were then mixed with protein A-Sepharose CL-4B beads (Pharmacia) previously bound with CYT-356 monoclonal antibody and rabbit anti-mouse IgG (Accurate Scientific) for 4 h at 4°C. Beads were then washed with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 2 mM sodium *o*-vanadate buffer, resuspended in Laemmli sample loading buffer, and denatured prior to electrophoresing on a 10% SDS-PAGE gel at 10 mA overnight. Gels were dried down at 60°C in a vacuum dryer and autoradiographed for 16–24 h at –70°C. For the large scale purification of 5–10 µg of PSM antigen, the above procedure was repeated using approximately 6 × 10⁷ LNCaP cells. The immunoprecipitation product was pooled and loaded into two lanes of a 10% SDS-PAGE gel and electrophoresed for 16 h at 10 mA. Proteins were electroblotted onto nitrocellulose membranes and stained with Ponceau red to visualize the proteins.

Peptide Microsequencing. This work was performed with the assistance of the Sloan-Kettering Institute Microchemistry Core Facility. Briefly, the *M*_r 100,000 PSM antigen band was excised from the membrane, solubilized, and digested proteolytically with trypsin. High performance liquid chromatography was performed on the digested sample using a HPLC Applied Biosystems Model 171C, and clear dominant peptide peaks were selected and sequenced on a modified post-liquid Applied Biosystems Model 477A Protein/Peptide Microsequencer (8). Nine peptides were sequenced ranging in size from 7 to 22 amino acids and all were screened for homology with the Genbank database and found to be unique. A similar technique was used to sequence the amino terminus of the PSM antigen and it was determined that it was in fact blocked, and no protein sequence was obtained.

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³The abbreviations used are: PAP, prostatic acid phosphatase; PSA, prostate-specific antigen; PSM, prostate-specific membrane glycoprotein; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; MSKCC, Memorial Sloan-Kettering Cancer Center; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

RNA Isolation. Total cellular RNA was isolated from LNCaP cells by standard techniques (9). Polyadenylate-enriched RNA was prepared from total RNA by oligo-deoxythymidylate cellulose chromatography (10).

PCR with Degenerate Primers. Sense and antisense 5'-unphosphorylated degenerate oligonucleotide primers 17 to 20 nucleotides long corresponding to portions of the previously sequenced peptides were synthesized on an Applied Biosystems Model 394A DNA Synthesizer. These primers consisted of mixtures of 32 to 144 different sequences, in order to account for the degeneracy of the genetic code. PCR (11) was performed on a Perkin-Elmer Model 480 DNA Thermal Cycler, using a cDNA template prepared by reverse transcribing LNCaP mRNA with Superscript reverse transcriptase (Gibco-BRL) according to the manufacturer's recommendations. The PCR profile used was 94°C for 30 s, 55°C for 1 min (varied with the T_m of the primers used), followed by 72°C for 2 min. This was carried out for 30 cycles. Reactions were performed in a total volume of 50 μ l containing 5 μ l 10 \times PCR buffer (166 mM NH_4SO_4 , 670 mM Tris, pH 8.8-2 mg/ml bovine serum albumin), 5 μ l 2.5 mM deoxynucleotide triphosphate mix, 5 μ l Primer mix (0.5–1.0 μ g each of sense and antisense primers), 5 μ l 100 mM β -mercaptoethanol, 2 μ l cDNA template, 5 μ l 25 mM MgCl_2 , 2 μ l diluted Taq polymerase at 0.5 unit/ μ l (Promega), and 21 μ l dH_2O .

Cloning of PCR Products. PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent *Escherichia coli* cells using standard methods (12) and plasmid DNA was isolated using Magic Minipreps (Promega) and screened by restriction analysis.

DNA Sequencing of PCR Products. TA clones were then sequenced by the dideoxy method (13) using Sequenase (United States Biochemical). From 3 to 4 μ g of each plasmid were denatured with NaOH and ethanol precipitated. Labeling reactions were carried out according to the manufacturer's recommendations using [32]dATP (NEN), and the reactions were terminated as discussed in the same protocol. Sequencing products were then analyzed on 6% polyacrylamide/7 M urea gels run at 120 W for 2 h. Gels were fixed for 20 min in 10% methanol/10% acetic acid, transferred to Whatman No. 3MM paper, and dried down in a vacuum dryer for 2 h at 80°C. Gels were then autoradiographed at room temperature for 16–24 h. Confirmation of correct clones was determined by reading DNA sequences adjacent to primer sequences looking for predicted peptide sequences that agreed with our peptide sequences.

cDNA Library Construction/Cloning of Full-Length cDNA. A cDNA library from LNCaP mRNA was constructed using the Superscript plasmid system (Gibco-BRL). The library was transformed using competent DH5- α cells (Gibco-BRL) and plated onto 100-mm plates containing L-Broth plus 100 μ g/ml of carbenicillin. Plates were grown overnight at 37°C and colonies were transferred to nitrocellulose filters. Filters were processed and screened following techniques described by Grunstein and Hogness (14), using the 1.1-kilobase partial cDNA homologous probe, radiolabeled with [32]dCTP by random priming (15). Positive colonies were sequenced by the Sequenase method as described previously.

Northern Analysis of PSM Gene Expression. Analysis of PSM mRNA was performed according to previously described techniques (16). Ten μ g of total RNA were denatured and electrophoresed through 1.1% agarose/formaldehyde gels at 60 mA for 8 h. RNA was then transferred to Nytran nylon membranes (Schleicher and Schuell) by pressure blotting in 10 \times standard saline-citrate with a Posi-blotter (Stratagene). RNA was cross-linked using a UV Stratalinker (Stratagene) and then baked in a vacuum oven for 2 h at 80°C. Blots were prehybridized at 65°C for 2 h and subsequently hybridized with denatured [32]P-labeled random-primed cDNA probe. Blots were washed twice in 1 \times saline-sodium phosphate-EDTA/0.5% SDS at 42°C and twice in 0.1 \times saline-sodium phosphate-EDTA/0.5% SDS at 50°C for 20 min each. Membranes were air-dried and autoradiographed for 12–36 h at -70°C with Kodak X-Omat film.

Results

Immunoprecipitation of the PSM Antigen. In agreement with previous results obtained by Western analysis using the CYT-356 monoclonal antibody (5), immunoprecipitation of the PSM antigen from metabolically labeled LNCaP cells yielded a single protein spe-

cies with an apparent molecular weight of 100,000 on SDS-PAGE electrophoresis (Fig. 1).

PSM Antigen Peptide Sequencing. Approximately 10 μ g of PSM antigen were purified as described in "Materials and Methods" and we obtained the following 9 peptide sequences:

1. SLYESWTK
2. SYPDGXNLPGGGVQR
3. FYDPMFK
4. IYNVIGTLK
5. FLYXXTQIPHLAGTEQNQLAK
6. GVILYSSDPADYFAPDGVK
7. AFIDPLGLPDRPFYR
8. YAGESFFGIYDALFDIESK
9. TILFASWDAEEFGXXGSTWAE

Each of these 9 peptide sequences was found within the predicted amino acid sequence translated from the PSM antigen cDNA with only a few minor changes, presumably due to limitations of the protein sequencing technology. An attempt was also made to sequence the amino terminus of the PSM antigen but no sequence data could be obtained and it was concluded that the amino terminus of the protein is blocked.

Polymerase Chain Reaction. Degenerate primers designed from peptides 5 and 9 listed above were used in the polymerase chain reaction to amplify a 1.1-kilobase partial cDNA which was confirmed correct by DNA sequencing by the identification of the above peptide sequences contained within it. This cDNA sequence was screened on the Genbank computer database (Los Alamos, NM) and was found to be unique.

Cloning of the Full-Length PSM Antigen cDNA. Using the 1.1-kilobase partial PSM cDNA as a hybridization probe, 4 cDNAs encoding the PSM antigen were detected in the LNCaP cDNA library. The complete sequence of the longest cDNA; clone 55A (2.65 kilobases) and its deduced protein sequence are shown in Fig. 2. The entire 1.1-kilobase partial cDNA sequence is contained within the full-length PSM cDNA without changes. The open reading frame is 750 amino acids with a predicted protein molecular weight of 84,000, excluding carbohydrate. The presence of 5 in-frame stop codons between nucleotides -120 and -94 indicates that the ATG at nucleotide +1 is probably the actual initiator codon. Partial sequence analysis of the other 3 cDNAs indicated that they are identical to clone 55A, except the 5' ends of these cDNAs terminate at different positions

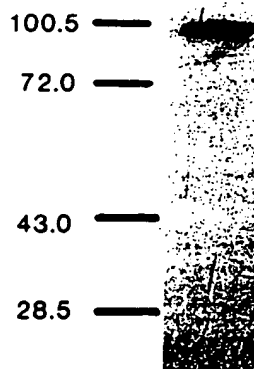


Fig. 1. Immunoprecipitation of the M_r 100,000 PSM antigen from [35 S]methionine-labeled LNCaP cells. Protein markers are shown on the left.

Fig. 2. Complete nucleotide sequence for the 2653-base pair PSM cDNA and the translated 750-amino acid predicted protein sequence. The transmembrane domain is double-underlined and polyadenylation signal is single-underlined. The area of homology with the human transferrin receptor is contained within the boxed region. Potential N-glycosylation sites are indicated with asterisks.

between the initiator codon and the 5' end of clone 55A. The sequences of two of these additional cDNAs extend upstream of the five in-frame stop codons; thus these stop codons are probably part of authentic PSM RNA and not a 5' artifact sequence. This provides further evidence that the coding region of PSM mRNA begins at nucleotide +1 of the sequence shown in Fig. 2.

Although the predicted protein sequence does not contain an NH₂-terminal hydrophobic signal sequence, a segment of hydrophobic residues from amino acids 20 through 43 forms a putative transmembrane domain (17). The major portion of the protein is COOH terminal to the transmembrane domain and contains multiple potential *N*-glycosylation sites. Greater than 54% homology on the nucleic acid level from nucleotides 1250 through 1700 within the coding region has been demonstrated with the human transferrin receptor mRNA by means of a Genbank homology search. Similar homologies have been identified for the chicken and rat transferrin receptor mRNAs. The entire full-length cDNA sequence has been compared to the Genbank database and confirmed to be unique.

Expression of the PSM Gene. Northern analysis using the PSM cDNA probe has revealed expression of a 2.8-kilobase message in the LNCaP cell line, with no expression in the DU-145 and PC-3 cell lines (Fig. 3). Expression of the PSM antigen appears to be limited to

prostatic tissues, both benign and neoplastic, with no detectable expression in any of the nonprostatic tissues and cell lines tested to date.⁴

Discussion

Organ-specific antigens permit insight into the processes that occur uniquely within a particular tissue. The prostate is a very unusual organ in that with aging most organs atrophy, whereas the prostate gland almost invariably hypertrophies and in a very high percentage of cases develops a malignancy. A cell model that has proven valuable in fostering our understanding of prostatic cancer has been the LNCaP prostate cancer cell line. These cells express markers characteristic of prostatic epithelial cells such as PSA and PAP as well as a functional androgen receptor. These cells were used to immunize mice and resulted in the generation of the 7E11-CS monoclonal antibody and its subsequent modified derivative, CYT-356, which we used to clone the PSM cDNA. The PSM antigen appears to have many interesting and potentially significant properties. The presence of 3 arginine residues at the NH₂-terminal end of the putative transmembrane domain suggests that the PSM protein is a type II integral membrane protein, with

⁴ Unpublished data.

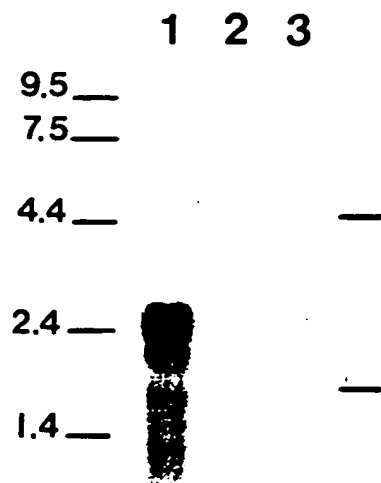


Fig. 3. Autoradiogram of Northern analysis revealing expression of 2.8-kilobase PSM message unique to the LNCaP cell line (Lane 1) and absent from the DU-145 (Lane 2) and PC-3 cell lines (Lane 3). RNA size ladder is shown on the left (kilobases), and 28S and 18S rRNA bands are indicated on the right.

a short NH₂-terminal region on the cytoplasmic side of the membrane and a large COOH-terminal domain on the extracellular side (17). This prediction is supported by the finding that removal of basic residues from the NH₂-terminal side of type II integral membrane protein transmembrane domains can reverse the orientation of such proteins in the membrane (18).

As an integral membrane protein unique to prostatic epithelial cells, the antigen or perhaps a specific PSM ligand may serve as an excellent site for use in the imaging and/or targeting of metastatic deposits. Indeed, current studies suggest that the CYT-356 antibody may be useful in imaging extraprostatic deposits of cancer cells (5). The CYT-356 antibody recognizes an epitope that is at least in part carbohydrate. It is possible that a unique peptide-recognizing antibody may have less nonspecific binding and that, additionally, multiple antibodies recognizing multiple areas of the PSM antigen may enhance the ability to image and treat metastatic prostate cancer.

PSA expression tends to decrease in hormone-refractory disease and bone metastases, while the expression of PSM appears to increase, again implying that it may provide an attractive target for therapy and diagnosis.

The homology to the human transferrin receptor is an interesting finding. It is of interest that the expressed prostatic secretions of patients with prostate cancer are enriched with respect to their content of transferrin and that prostatic cancer cells are rich in transferrin receptors (19). It was previously hypothesized that the microenvironment of bone would serve to stimulate prostatic cancer cell growth. This was recently observed to be the case, inasmuch as bone stroma cell transferrin dramatically stimulated the growth of metastatic prostatic cancer cell lines (20). In these experiments, the androgen receptor-negative DU-145 and PC-3 cell lines were used and LNCaP cells were not examined. Whether the PSM antigen interacts with

transferrin or another ligand and possibly facilitates metastatic spread is presently being addressed in our laboratory. Transferrin may prove to be more than a transport molecule, because apotransferrin has been shown to be mitogenic to some tumor cells (21).

Finally, we are presently developing new antibodies directed against peptide epitopes of the PSM antigen which are predicted to be highly antigenic, with the expectation that these may be used to develop serum enzyme-linked immunosorbent assays, aid in tissue diagnoses, and serve as new agents for the immunotherapy of advanced, hormone-refractory prostate cancer.

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References

- Chiarodo, A. National Cancer Institute roundtable on prostate cancer: future research directions. *Cancer Res.* 51: 2498-2505, 1991.
- Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. P. LNCaP model of human prostatic carcinoma. *Cancer Res.* 43: 1809-1818, 1983.
- Horoszewicz, J. S., Kawinski, E., and Murphy, G. P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. *Anticancer Res.* 7: 927-936, 1987.
- Axelrod, H. R., Gilman, S. C., D'Aleo, C. J., Petrylak, D., Reuter, V., Gulfo, J. V., Saad, A., Cordon-Cardo, C., and Scher, H. I. Preclinical results and human immunohistochemical studies with ⁹⁰Y-CYT-356: a new prostatic cancer therapeutic agent. *AUA Proceedings*, Abstract 596, 1992.
- Abdel-Nabi, H., Wright, G. L., Gulfo, J. V., Petrylak, D. P., Neal, C. E., Texter, J. E., Begun, F. P., Tyson, I., Heal, A., Mitchell, E., Purnell, G., and Harwood, S. J. Monoclonal antibodies and radioimmunoconjugates in the diagnosis and treatment of prostate cancer. *Semin. Urol.* 10: 45-54, 1992.
- Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., and Paulson, D. F. Isolation of a human prostate carcinoma cell line (DU-145). *Int. J. Cancer* 21: 274-281, 1978.
- Kaign, M. E., Narayan, K. S., Ohnuki, Y., and Lechner, J. F. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest. Urol.* 17: 16-23, 1979.
- Tempst, P., and Riviere, L. Examination of automated polypeptide sequencing using standard phenyl isothiocyanate reagent and subpicomole high-performance liquid chromatographic analysis. *Anal. Biochem.* 183: 290-300, 1989.
- Glisin, V., Crkvenjakov, R., and Byus, C. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* 13: 2633-2637, 1974.
- Aviv, H., and Leder, P. Purification of biologically active globin messenger RNA by chromatography on oligo-thymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA* 69: 1408-1412, 1972.
- Lee, C. C., Wu, X., Gibbs, R., Cook, R. G., Muzny, D. M., and Caskey, C. T. Generation of cDNA probes directed by amino acid sequence: cloning of urate oxidase. *Science (Washington DC)* 239: 1288-1292, 1988.
- Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-580, 1983.
- Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463-5467, 1977.
- Grunstein, M., and Hogness, D. S. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* 72: 3961-3965, 1975.
- Feinberg, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132: 6-13, 1983.
- Rave, N., Crkvenjakov, R., and Boedtker, H. Identification of procollagen mRNAs transferred to diazobenzylmethyl paper from formaldehyde agarose gels. *Nucleic Acids Res.* 6: 3559-3567, 1979.
- von Heijne, G. Transcending the impenetrable: how proteins come to terms with membranes. *Biochim. Biophys. Acta* 947: 307-333, 1988.
- Parks, G. D., and Lamb, R. A. Topology of eukaryotic type II membrane proteins: importance of N-terminal positively charged residues flanking the hydrophobic domain. *Cell* 64: 777-787, 1991.
- Keer, W. P., Koslowski, J. M., Tsai, Y. C., Lee, C., McEwan, R. N., and Grayhack, J. T. Elevated transferrin receptor content in human prostate cancer cell lines assessed *in vitro* and *in vivo*. *J. Urol.* 143: 381-385, 1990.
- Rossi, M. C., and Zetter, B. R. Selective stimulation of prostatic carcinoma cell proliferation by transferrin. *Proc. Natl. Acad. Sci. USA* 89: 6197-6201, 1992.
- Sirbasku, D. A., Pakala, R., Sato, H., and Eby, J. E. Purification of an equine apotransferrin variant (thyromedin) essential for thyroid hormone dependent growth of GH₁ rat pituitary tumor cells in chemically defined culture. *Biochemistry* 30: 7466-7477, 1991.

Vascular and interstitial barriers to delivery of therapeutic agents in tumors

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Abstract

The efficacy in cancer treatment of novel therapeutic agents such as monoclonal antibodies, cytokines and effector cells has been limited by their inability to reach their target *in vivo* in adequate quantities. Molecular and cellular biology of neoplastic cells alone has failed to explain the nonuniform uptake of these agents. This is not surprising since a solid tumor *in vivo* is not just a collection of cancer cells. In fact, it consists of two extracellular compartments: vascular and interstitial. Since no blood-borne molecule or cell can reach cancer cells without passing through these compartments, the vascular and interstitial physiology of tumors has received considerable attention in recent years. Three physiological factors responsible for the poor localization of macromolecules in tumors have been identified: (i) heterogeneous blood supply, (ii) elevated interstitial pressure, and (iii) large transport distances in the interstitium. The first factor limits the delivery of blood-borne agents to well-perfused regions of a tumor; the second factor reduces extravasation of fluid and macromolecules in the high interstitial pressure regions and also leads to an experimentally verifiable, radially outward convection in the tumor periphery which opposes the inward diffusion; and the third factor increases the time required for slowly moving macromolecules to reach distal regions of a tumor. Binding of the molecule to an antigen further lowers the effective diffusion rate by reducing the amount of mobile molecule. Although the effector cells are capable of active migration, peculiarities of the tumor vasculature and interstitium may be also responsible for poor delivery of lymphokine activated killer cells and tumor infiltrating lymphocytes in solid tumors. Due to micro- and macroscopic heterogeneities in tumors, the relative magnitude of each of these physiological barriers would vary from one location to another and from one day to the next in the same tumor, and from one tumor to another. If the genetically engineered macromolecules and effector cells, as well as low molecular weight cytotoxic agents, are to fulfill their clinical promise, strategies must be developed to overcome or exploit these barriers. Some of these strategies are discussed, and situations wherein these barriers may not be a problem are outlined. Finally, some therapies where the tumor vasculature or the interstitium may be a target are pointed out.

Introduction

The advent of hybridoma technology and genetic engineering has led to the design and large-scale production of monoclonal antibodies (MAbs) and other biological macromolecules potentially useful for cancer detection and treatment. These mole-

cules can be conjugated to radionuclides, chemotherapeutic agents, toxins, growth factors, enzymes, effector cells or liposomes. Moreover, a number of genetically engineered cytolytic macromolecules (e.g., cytokines) and killer cells are under active investigation as potential therapeutic agents. While the concept of using antibodies, cy-

tokines or effector cells with a high degree of specificity for cancer cells remains attractive for cancer therapy, clinical results have not, to date, lived up to the earlier promises of their perceived potential. Similarly, many of the conventional drugs of lower molecular-weight, although effective against hematologic cancers (e.g., leukemias, lymphomas), have had minimal impact on solid tumors (e.g., breast, lung, colon, brain). A key problem with blood-borne therapeutic agents is their inability to reach all regions of a tumor in adequate quantities [1]. Cellular factors (e.g., heterogeneity of tumor-associated antigen, multidrug resistance) *alone* can not account for the poor delivery of these agents in tumors. This is not surprising since a solid tumor is a pathophysiological entity, which is more than the sum of its component cells. This tissue is capable of behaving as differently from its component cells, much as the liver does when compared with hepatocytes [2]. The neoplastic cells *in vivo* are, in fact, part of an organized structure which has at least two extracellular compartments: the vasculature and the interstitium [2]. While much of cancer biology has advanced rapidly in recent years, the study of tumor pathophysiology of cancer has lagged behind.

A blood-borne molecule or cell that enters the tumor vasculature reaches cancer cells via: (a) distribution through the vascular compartment; (b) transport across the microvascular wall; and (c) transport through the interstitial compartment. For a molecule of given charge, size, and configuration, each of these transport processes may involve convection (i.e., solute movement associated with bulk solvent movement) and diffusion (i.e., solute movement resulting from solute concentration gradients). In addition, during this journey the molecule may bind non-specifically to proteins or other tissue components; bind specifically to the target(s) and/or be metabolized [3, 4]. Although LAK or TIL cells are capable of active migration, they encounter the same barriers against their movement in tumors. In this article, I will critically review these physiological barriers to delivery of molecules and cells in tumors and discuss some strategies to overcome or exploit them for therapeutic benefit.

Distribution through vascular space

The tumor vasculature consists of (a) vessels recruited from the preexisting network of the host vasculature and (b) vessels resulting from the angiogenic response of host vessels to cancer cells [5, 6]. Movement of molecules through the vasculature is governed by the vascular morphology (i.e., the number, length, diameter and geometrical arrangement of various blood vessels) and the blood flow rate.

Vascular morphology

Although the tumor vasculature originates from the host vasculature, its organization may be completely different depending upon the tumor type, its growth rate, and its location. The architecture is different not only among various tumor types, but also between a spontaneous tumor and its transplants [7].

Macroscopically, the tumor vasculature can be studied in terms of two idealized categories: peripheral and central. In tumors with peripheral vascularization, the centers are usually poorly perfused (Fig. 1). In those with central vascularization, one would expect the opposite. Hence, the penetration of blood-borne substances should follow the same pattern. In reality, a tumor may consist of many territories, each exhibiting one or the other of these two types of idealized vascular patterns.

Microscopically, the tumor vasculature is highly heterogeneous and does not conform to the standard normal vascular organization (i.e., artery to arteriole to capillaries to postcapillary venule to venule to vein). Based on their ultrastructure, the tumor vessels can be classified into nine categories: a) arteries and arterioles; b) nonfenestrated capillaries; c) fenestrated capillaries; d) discontinuous capillaries (sinusoids); e) blood channels without endothelial lining; f) capillary sprouts; g) postcapillary venules (giant capillaries); h) venules and veins; and i) arteriovenous anastomoses (shunts) [7]. Note that except for vessels of classes (e) and (f), the remaining vessel types are structurally similar to those found in a normal tissue. The vessels of

classes (e) and (f) are found in healing (granulation) tissue. A key difference between normal and tumor vessels is that the latter are dilated, saccular and tortuous, and may contain tumor cells within the endothelial lining of the vessel wall [7]. In addition, unlike a normal tissue with a fixed route between arterial and venous sides, a tumor may have blood flowing from one venule to another via vessels of classes (b) through (g), or directly via an arteriovenous shunt. The branching patterns of blood vessels in a tumor are significantly different from those in a normal tissue, with many trifurcations, self-loops and spouts [8]. Furthermore, due to the peculiar nature of the vasculature, the organization of vessels may be different from one location to another and from one time to the next. As a result, one would expect different routes for blood flow in the well perfused advancing zone, semi-necrotic zone, and necrotic zone (Fig. 1).

Following the pioneering studies of Algire [9], several investigators have measured morphometric parameters of vessels in thin, two-dimensional tumors grown in transparent windows. The pioneering work of Gullino and Grantham [10] led to similar studies in three-dimensional experimental and human tumors [7]. The vascular space in tumors varies from 1% to 20% depending upon the tumor type, weight and method of measurement. Studies in two-dimensional tumors show that vascular volume, length and surface area increase during the early stages of growth, and then decrease; this behavior correlates with the onset of necrosis. The frequency of large diameter vessels increases in the later stages of growth. Most quantitative studies in three-dimensional tumors miss the early growth period of increase in vascular volume, length and surface area. While studies of later stages of growth show an increase in the intercapillary distance and a decrease in vessel length and surface area, the results on vascular volume are inconclusive. Some studies show that the fractional vascular volume of tumors remains fairly constant during growth (suggesting an increase in the number of blood vessels with sluggish flow), while others show that the fractional vascular volume decreases as a tumor grows (in agreement with the observation that tumor perfusion rate decreases as a tumor grows) [7].

Possible reasons for this discrepancy include errors associated with different measurement techniques as well as presence of arteriovenous shunts and blood vessels with stagnant blood in them. Whether the vascular volume decreases or not, a reduction in vascular surface area would lead to a reduction in the transvascular exchange of molecules. In addition, an increase in the intercapillary distance would require the molecules to traverse longer distances in the interstitium to reach all regions of a tumor.

Blood flow rate

Most investigators have measured local blood flow rate of tumors based on uptake or clearance of a tracer from a single or a limited number of regions of the tumor. Due to noticeable spatial and temporal heterogeneity in tumor blood supply, these values may not be representative of the whole tumor. A limited number of studies in which the blood flow rate of the whole tumor has been measured, shows that the *average* perfusion rate of carcinomas is less than that of the host tissue of origin. Sarcomas and lymphomas have higher *average* perfusion rates than carcinomas [11]. The data on blood flow in human tumors is limited and inconclusive due to methodological problems [12]. In general, as tumors grow larger, they may develop necrotic foci, and as a result, the average perfusion rate decreases with tumor size [11]. Note that even in these large necrotic tumors, therapeutic agents would be delivered in the well perfused regions.

Since the seminal work of Ide *et al.* [13], several investigators have examined the microscopic flow heterogeneities of tumors grown in transparent windows. Blood flow in tumor vessels has been found to be intermittent. There are random periods of flow reduction and stasis followed by resumption of flow, sometimes in the opposite direction [14, 15]. These fluctuations may result from a) vasomotor activity of the host arterioles, b) respiratory or cardiac cycle, c) rheological factors such as passage of red blood cells, white blood cells or cancer cells in a vessel, d) low perfusion pressures

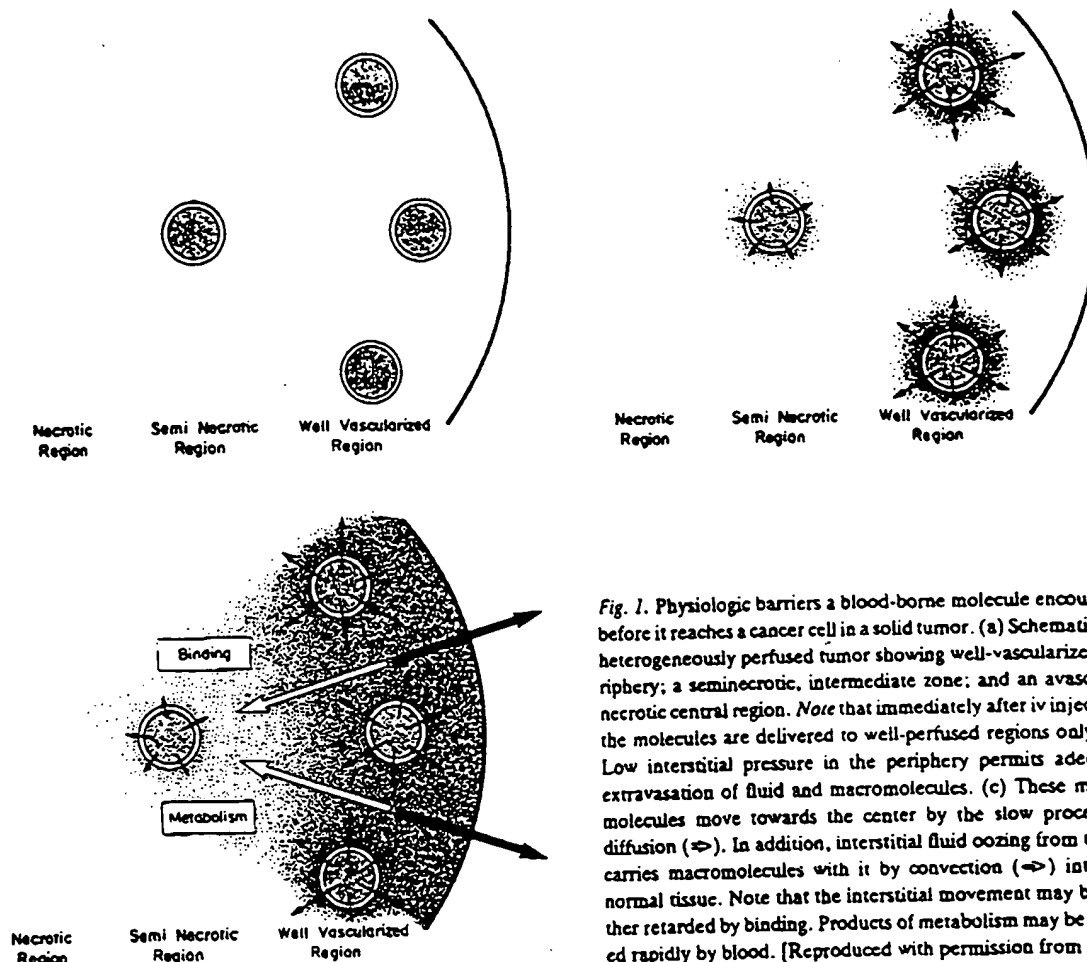


Fig. 1. Physiologic barriers a blood-borne molecule encounters before it reaches a cancer cell in a solid tumor. (a) Schematic of a heterogeneously perfused tumor showing well-vascularized periphery; a seminecrotic, intermediate zone; and an avascular, necrotic central region. Note that immediately after iv injection, the molecules are delivered to well-perfused regions only. (b) Low interstitial pressure in the periphery permits adequate extravasation of fluid and macromolecules. (c) These macromolecules move towards the center by the slow process of diffusion (\rightleftharpoons). In addition, interstitial fluid oozing from tumor carries macromolecules with it by convection (\Rightarrow) into the normal tissue. Note that the interstitial movement may be further retarded by binding. Products of metabolism may be cleared rapidly by blood. [Reproduced with permission from [1].]

in tumor vessels, and/or e) elevated interstitial pressure in tumors [7, 16, 17].

Quantitative studies on the macroscopic spatial heterogeneities in the tumor perfusion rate as a function of tumor growth (size) are limited. Based on perfusion rates four regions can be recognized in a tumor: a) an avascular, necrotic region, b) a seminecrotic region, c) a stabilized microcirculation region and d) an advancing front. In a rhabdomyosarcoma grown in the transparent chamber in a rat, the widths of the stabilized region and the advancing front were found to remain constant, while the widths of the necrotic and the seminecrotic zones increased with tumor growth. In addition, the perfusion rate in the tumor periphery (i.e., the

stabilized and advancing zones) was found to be higher than that in the surrounding normal tissue [14]. Intratumor blood flow distributions in spontaneous animal and human tumors are now being investigated using nuclear magnetic resonance and positron emission tomography. While limited, these results are in concert with the transplanted tumor studies: blood flow rates in necrotic/seminecrotic regions of tumors are low, while those in non-necrotic regions are variable and substantially higher than in surrounding/contralateral host normal tissues [18, 19]. As a result of these spatial and temporal heterogeneities in blood supply coupled with variations in the vascular morphology at both macroscopic and microscopic levels, it is not sur-

prising that the spatial distribution of therapeutic agents in tumors is heterogeneous and the average uptake decreases with an increase in tumor weight.

Transport across microvascular wall

Once a blood-borne molecule has reached an exchange vessel, its extravasation, J_s (g/s), occurs by diffusion and convection (Fig. 2) and, to some extent, by transcytosis. Diffusion is proportional to the exchange vessel's surface area, S (cm²), and the difference between the plasma and interstitial concentrations ($C_p - C_i$; g/ml). Convection is proportional to the rate of fluid leakage, J_f (ml/s), from the vessel. J_f , in turn, is proportional to S and the difference between the vascular and interstitial hydrostatic pressures ($p_v - p_i$; mm Hg) minus the difference between the vascular and interstitial osmotic pressure ($\pi_v - \pi_i$; mm Hg). The proportionality constant which relates transmural diffusive flux to concentration gradients ($C_p - C_i$) is referred to as the vascular permeability, P (cm/s), and the constant which relates fluid leakage to pressure gradients is referred to as the hydraulic conductivity, L_p (cm/mm Hg-s). The effectiveness of the transmural osmotic pressure difference in producing fluid movement across a vessel wall is characterized by the osmotic reflection coefficient, σ ; σ is close to 1 for a macromolecule and close to zero for a small molecule [20]. Thus, transport of a molecule across normal or tumor vessels is governed by three transport parameters, P , L_p , and σ ; the surface area for exchange, S ; and the transvascular concentration and pressure gradients.

Transvascular transport parameters

For a macromolecule of specified size, charge, configuration and binding constants, the transport parameters depend upon the physiological properties of the vessel wall (e.g., wall structure, charge). Ultrastructural studies of animal and human tumors have shown that tumor vessels have wide interendothelial junctions, large number of fen-

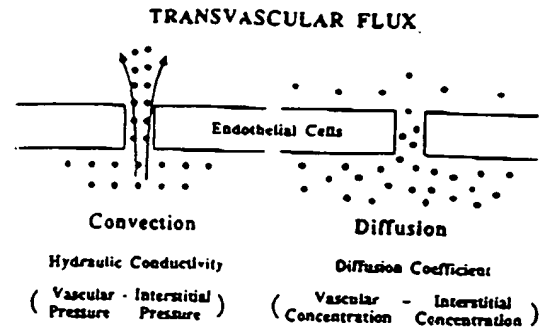


Fig. 2. Two primary modes of transvascular transport of molecules. Diffusion is proportional to concentration gradients and convection is proportional to pressure gradients. For more details on other modes of transport, see the text and [20].

estrae and transendothelial channels formed by vesicles, discontinuous or absent basement membrane and a significant spatial heterogeneity [20, 21]. These characteristics of tumor vessels suggest that they should have relatively high P and L_p [22, 23]. As a matter of fact, various tissue uptake studies have found vascular permeability of tumors to be significantly higher than that of skin or muscle (Fig. 3; [20]). If tumor vessels are indeed 'leakier' to fluid and macromolecules compared to several normal tissues, what leads to their poor extravasation? As discussed below, tumors contain regions of high interstitial pressure, which lowers the fluid extravasation. Since the transvascular transport of macromolecules under normal conditions occurs primarily by convection [20], a decrease in fluid extravasation would lead to a decrease in extravasation of macromolecules [25-27]. Furthermore, the average vascular surface area decreases with tumor growth, hence one would expect reduced transvascular exchange in large tumors compared to smaller tumors [27].

Transvascular pressure gradients

Decreased p_v and/or increased p_i in tumors has been indirectly demonstrated by several investigators working with tumors grown in transparent chambers. By raising venous pressure in the cham-

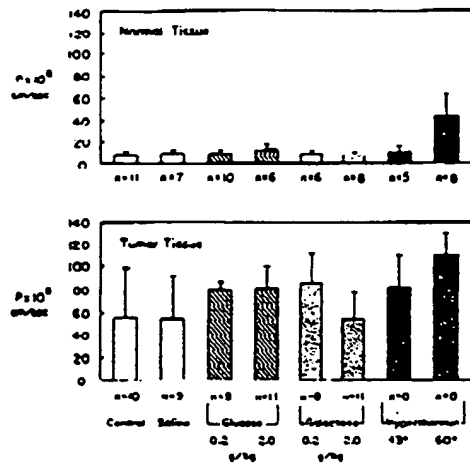


Fig. 3. Effective microvascular permeability coefficient of 150,000-molecular-weight dextran in normal (manure granulation) and neoplastic (VX2 carcinoma) tissues under various conditions: (a) control [37]; (b) after saline (1 ml/kg of body weight) injection; (c) after glucose injections; (d) after galactose injections; and (e) after hypotonicity for 1 hr [23] (mean \pm SD; n = No. of measurements). Note that these measurements were made in individual blood vessels, and that tumor exchange vessels exhibit tremendous heterogeneity in vascular permeability. In addition, IgGs may extravasate at rates different from dextrans (Claus M. Jais RK, unpublished results). [Reproduced with permission from [24].]

ber or by loosening the chamber, blood flow can be restored in ischemic/necrotic tumor areas. Direct measurements in sandwich tumors or in the superficial layer of three-dimensional tumors have shown that on the arterial side vascular pressure does not differ significantly between non-tumor and tumor vessels; whereas, venous pressures may be lower in tumor vessels compared to those in normal vessels [7].

Since the initial work of Young *et al.* [28], several investigators have shown that p_i in animal tumors is significantly higher than in normal tissues [29]. Recently, we have also quantified interstitial hypertension in human tumors. [For example, in superficial melanomas p_i is as high as 45 mm Hg;¹ in cervical carcinomas as high as 30 mm Hg.²] Further as the tumor grows, p_i rises in some tumors, presumably due to the proliferation of tumor cells in a confined space, high vascular permeability, and the

absence of functioning lymphatic vessels [29–32]. This increase in p_i also correlates with a reduction in tumor blood flow and the development of necrosis in a growing tumor [29]. Recent investigations of intratumor pressure gradients show that the interstitial pressure is elevated throughout the tumor and its drops precipitously to normal physiological values in the tumor periphery (Fig. 4; [30]). This pressure profile is in agreement with the predictions of our mathematical model [25–27].

In normal tissues π_i and π_e are approximately 20–25 and 5–15 mm Hg, respectively [25, 26]. Although there are no direct measurements of π_i in tumors, based on high vascular permeability and high interstitial diffusion coefficient in tumors, one would expect higher concentration of endogenous plasma proteins in the tumor interstitium than in normal interstitium. This hypothesis is supported by the data in the literature [33]. As a result, π_i in tumors may be higher than that in normal tissues, and may lead to reduced osmotic flow.

As shown in Fig. 4, p_i in tumors is close to zero in the periphery, therefore the filtration of fluid from vessels, J_f , would be close to normal. However, as one moves towards the center of the tumor, the increase in p_i would reduce the extravasation of fluid, J_f . As stated earlier, convective transport of a macromolecule is proportional to J_f , therefore, the rate of extravasation of a blood-borne macromolecule would be negligible in the center of a tumor [25, 26]. Since transvascular transport by diffusion is negligible for a macromolecule to begin with, macromolecular extravasation would be very small in the high interstitial pressure regions of a tumor. Since high pressure regions usually coincide with regions of poor perfusion rate and lower vessel surface area, leakage of blood-borne macromolecules from vessels would be further restricted [27].

Transport through interstitial space

Once a macromolecule has extravasated, its movement occurs by diffusion and convection through the interstitial space [34]. Diffusion is proportional to the concentration gradient in the interstitium, and convection is proportional to the interstitial

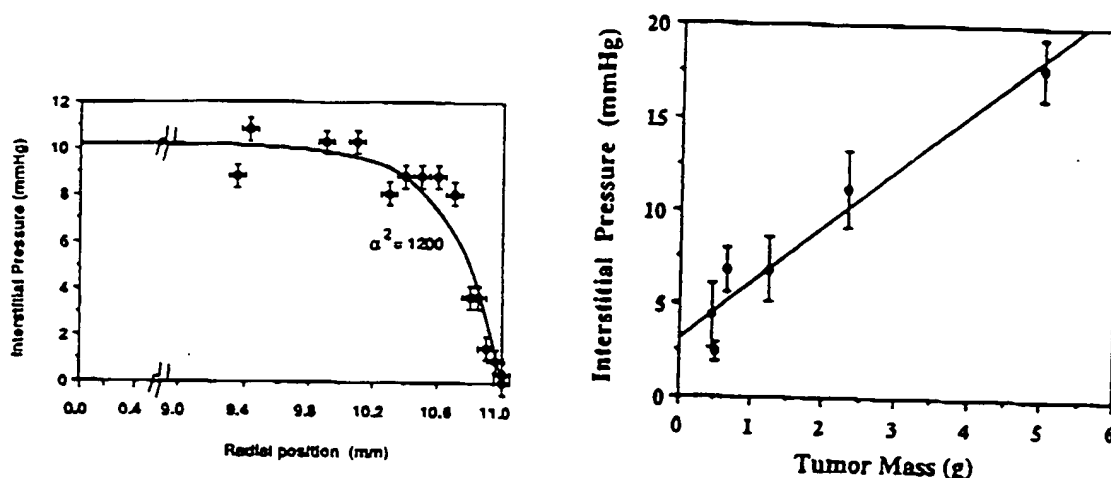


Fig. 4. (a) Interstitial pressure gradients in the mammary adenocarcinoma R3230AC as a function of radial position. The circles (●) represent data points [30] and the solid line represents the theoretical profile based on our previously developed mathematical model [25, 26]. Note that the pressure is nearly uniform in most of the tumor, but drops precipitously to normal tissue values in the periphery. (b) In Walker 256 carcinoma grown as tissue-isolated tumor, the mean central pressure (\pm SD) is linearly related to the tumor weight (pressure = $3.05 \times \text{weight (g)} + 3.02 \text{ mm Hg}$). However, not all tumor types exhibited such correlation. In some tumors, the interstitial pressure increased initially with tumor growth and reached a maximum value (Belton A, Jain RK, unpublished results). We have also measured interstitial pressure as high as 45 mm Hg in human tumors (see text). Note that elevated pressure in the central region retards the extravasation of fluid and macromolecules. In addition the pressure drop from the center to the periphery leads to an experimentally verifiable, radially outward fluid flow. [Reproduced from [30], with permission.]

fluid velocity, u_i (cm/s). The latter, in turn, is proportional to the pressure gradient in the interstitium. The proportionality constant which relates diffusive flux to the concentration gradient is referred to as the interstitial diffusion coefficient, D (cm^2/s), and the constant which relates u_i to the pressure gradient is referred to as the interstitial hydraulic conductivity, K ($\text{cm}^2/\text{mm Hg}\cdot\text{s}$) [29]. Values of transport coefficients D and K are determined by the structure and composition of the interstitial compartment as well as the physicochemical properties of the solute molecule. Larger values of these parameters lead to less hindered movement of fluid and macromolecules through the interstitium. Similarly, large values of interstitial pressure and concentration gradients lead to large convective and diffusive fluxes.

Interstitial transport coefficients

The interstitial space in tumors, in general, is very large compared to that in host normal tissues [29].

Similar to normal tissues, the interstitial space of tumors is composed predominantly of a collagen and elastic fiber network. Interdispersed within this cross-linked structure are the interstitial fluid and macromolecular constituents (polysaccharides) which form a hydrophilic gel. While collagen and elastin impart structural integrity to a tissue, the polysaccharides (glycosaminoglycan and proteoglycans) are presumably responsible for the resistance to fluid and macromolecular motion in the interstitium. In several tumors studied to date, collagen content of tumors is higher than that of the host normal tissue. On the other hand, hyaluronate and proteoglycans are, in general, present in lower concentrations in tumors than in the host normal tissue [29]. The lower concentration of these polysaccharide molecules is presumably due to increased activity of lytic enzymes, e.g., hyaluronidase, in the tumor interstitial fluid [5].

The large interstitial space and low concentrations of polysaccharides suggest that values of K and D should be relatively high in tumors. As a matter of fact, the data on hydraulic conductivity of

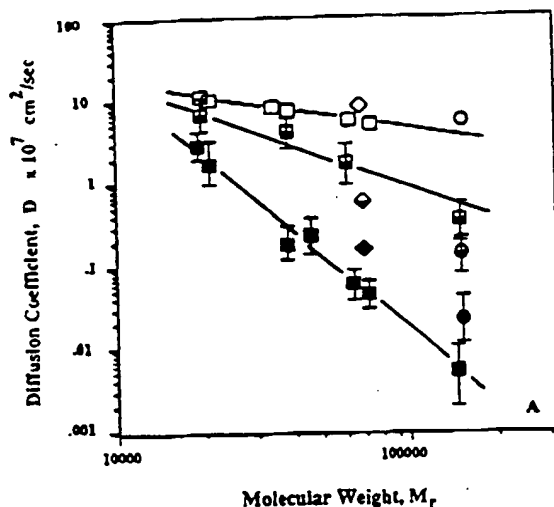


Fig. 5. Molecular weight dependence of effective diffusion coefficients, D , of dextrans [36, 37], bovine serum albumin [36] and rabbit IgG [38] in water, tumor, and normal (mature granulosa) tissue. Note that transport is hindered in both tissues compared with water and that there is tremendous heterogeneity in both tissue types. Despite higher values of D in tumors compared with nontumor tissues, macromolecules do not reach uniform concentration in a large tumor for a long time because of large diffusion distances and large size of molecules. Note that the proteins show the same trend, i.e., the interstitial diffusion coefficient is higher in normal tissue and lower in tumor tissue than the corresponding molecular weight dextrans. The symbols represent:
 □ Dextran-Water ◇ Albumin-Water ○ IgG-Water
 ■ Dextran-Tumor ◆ Albumin-Tumor ● IgG-Tumor
 ■ Dextran-Normal ◆ Albumin-Normal ● IgG-Normal
 [Reproduced from [36] with permission.]

hepatoma 5123 [35] and the data on effective diffusion coefficients of various macromolecules in VX2 carcinoma (Fig. 5) [36–38] support this hypothesis. An order of magnitude higher values of D and K in tumors compared to several normal tissues should favor movement of macromolecules in the tumor interstitium. Then, why do the exogenously injected macromolecules not distribute uniformly in tumors? As discussed below, there are two reasons for this apparent paradox:

Large distances in the interstitium

The time constant, τ_0 , for a molecule, with diffusion coefficient D , to diffuse across distance l is approximately $l^2/4D$. For diffusion of IgG in tumors (using D from Fig. 5), τ_0 is of the order of 1 hour for 100 μm distance, ~ 2 days for 1 mm distance and ~ 7 –8 months for 1 cm distance [25, 38] (Fig. 6). These numbers are consistent with the data on the penetration of MAbs in spheroids [39]. Now consider a hypothetical tumor which is uniformly perfused, has nearly zero p_i , and has exchange vessels $\sim 200 \mu\text{m}$ apart. In such a tumor, IgG would reach uniform concentration in ~ 1 hour post injection provided the plasma concentration remains constant. In a normal tissue with the value of D lower by an order of magnitude (Fig. 5), it will take ~ 10 hours to reach $\sim 16\%$ concentration.

Now consider a more realistic situation, where the tumor vessels are $\sim 200 \mu\text{m}$ apart and uniformly perfused, but p_i has increased in the center so that fluid extravasation, and, hence, convective transport of macromolecules across vessels has stopped. In such a case the only way macromolecules extravasate in the center is by the slow process of diffusion across vessel walls. Also they can reach the center from the periphery (where p_i is near zero) by interstitial diffusion. As stated earlier, if the distance between the center and periphery is ~ 1 mm, it would take days for them to get there and if it is ~ 1 cm, it would take months [25, 38]. If due to elevated p_i and cellular proliferation, the central vessels have collapsed completely, then there is no delivery of macromolecules by blood flow to the necrotic center [7]. In such a case, there are no molecules available for extravasation by diffusion across the vessel wall, and consequently the central concentration would be even lower [27].

So far the interstitial movement of molecules which do not bind to any extravascular sites or undergo metabolism has been discussed. It is well known that the binding reaction lowers the apparent diffusion rate of molecules [40]. Therefore, although higher affinity of antibody to antigen significantly increases the antibody's concentration proximal to the vessel, it retards their movement to

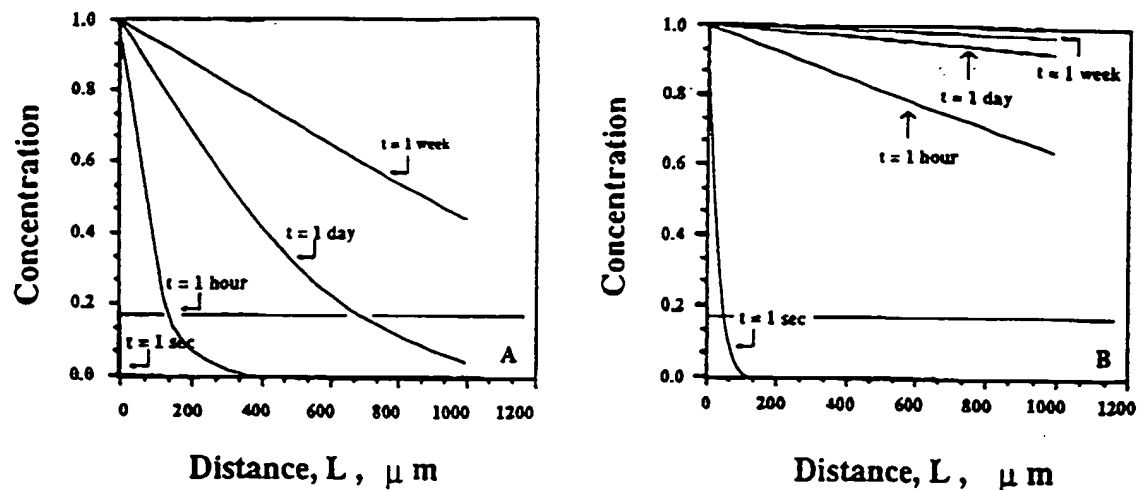


Fig. 6. The concentration profile of (a) IgG and (b) a low molecular weight agent ($M_r = 400$; $D_{\text{inter}} = 6.4 \times 10^{-4} \text{ cm}^2/\text{sec}$ [36]) at various distances as a function of time into the tumor interstitium using a one-dimensional model. These profiles assume a constant source of the drug outside the blood vessel ($L = 0$). Note that in a short time (less than an hour) a large fraction of the low M_r molecules has diffused to distances of 1 mm. On the other hand, for IgG, the fraction of antibody arriving at this point in 1 hour is nearly zero. The horizontal dotted lines show the time required to reach $\sim 16\%$ of the source concentration at distance L from the vessel wall. [Reproduced from [36] with permission.]

distal locations in the interstitium unless the antigens are saturated [41–44]. The metabolism of antibodies in normal and tumor tissues is poorly understood. However, the products of metabolism are usually smaller in molecular weight, and hence may be cleared relatively rapidly [43].

Interstitial fluid loss from tumor's periphery

It is a well known law of physics that fluid flows from a high to a low pressure region. As discussed earlier, p_i is high in the center of tumors and low in the periphery. Therefore one would expect interstitial fluid motion from the center of a tumor towards its periphery from where it will ooze out into the surrounding normal tissue. Using a tissue isolated tumor preparation, Butler *et al.* [45] measured this fluid loss to be 0.14–0.22 ml/h/g-tissue in four different rat mammary carcinomas. In various animal and human (xenograft) tumors studied to date, 1–14% of plasma entering the tumor has been found to leave from the tumor's periphery [22, 45–47]. This fluid leakage leads to a radially outward interstitial fluid velocity of 0.1–0.2 $\mu\text{m/s}$ at the

periphery of a 1 cm 'tissue-isolated' tumor [20]. [The radially outward velocity is an order of magnitude lower in a tumor grown in the subcutaneous tissue or muscle [25–26].] A macromolecule at the tumor periphery has to overcome this outward convection to penetrate into the tumor by diffusion. The relative contribution of this mechanism of heterogeneous distribution of antibodies in tumors is, however, smaller than the contribution of heterogeneous extravasation due to elevated pressure and necrosis [25–27, 43].

Conclusions and strategies for improved delivery

Antibodies linked to radionuclides, drugs, toxins, enzymes, growth factors and effector cells offer a promising approach to the treatment of solid tumors. Their strengths include their high degree of specificity for tumor-associated antigens and the fact that exchange vessels and interstitium of tumors are more 'leaky' to macromolecules than those of several normal tissues. Their clinical limitation, however, results from their inadequate uptake and non-optimal distribution in tumors. The

physiological factors which contribute to the poor delivery in tumors include – heterogeneous blood supply, interstitial hypertension, and relatively long transport distances in the interstitium (Table 1). How can these physiological barriers be overcome?

Several physical (e.g., radiation, heat) and chemical (e.g., vasoactive drugs) agents may lead to an increase in tumor blood flow or vascular permeability [7, 11, 48]. A key problem with this approach is that the increase in blood flow is short-lived and usually confined to well-vascularized regions. Increased delivery of macromolecules to well-perfused regions may not solve the maldistribution problem. However, an increase in the diffusive component of vascular permeability caused by these agents may increase the antibody uptake [49, 50].

The second approach may be based on lowering the tumor interstitial pressure. The interstitial hypertension results presumably from interstitial fluid accumulation which, in turn, results from the lack of functioning lymphatics in tumors [5, 25, 29, 30]. Since K is a key determinant of interstitial fluid motion, any method which increases K may lower pressure. Use of lytic enzymes (e.g., hyaluronidase) to increase K is one possibility [35]. An alternate strategy would be to lower the tumor cell density without destroying the vasculature. Whether fractionated radiation or other therapies (e.g. TNF) lowers p_i in tumors via this mechanism remains a plausible hypothesis to be tested [20, 25]. [In support of this hypothesis, we have recently found that in human cervical carcinomas, the pres-

sure decreased during fractionated radiation treatment in some patients.^{2]} The use of an osmotic agent (e.g. mannitol) may increase π , and hence increase antibody penetration [51]. However, this increase must have a long duration to yield practical results.

The third approach may be based on increasing the interstitial transport rate of molecules. Use of cocktails of antibodies may not overcome this problem because each antibody has to cross the same physiological barriers. One method of accomplishing this goal would be to use lower molecular weight agents, e.g., antibody fragments $F_{(ab)_2}$ and F_{ab} . While the fragments have higher values of P and D compared to the intact antibody and hence, penetrate deeper into tumors, there are two physiological problems associated with their use – they are eliminated more rapidly from blood, and their uptake into normal tissues is also increased. The elimination problem can be overcome by repeated or continuous injections of non-immunogenic fragments of chimeric or human antibodies. However, as the molecular weight is lowered further, the normal tissue toxicity problem may become more pronounced similar to that encountered with conventional anticancer agents (molecular weight < 2,000) [52, 53]. Some of the problems with the systemic toxicity may be overcome by local injection (e.g., intra-arterial, interstitial, intraperitoneal) at the cost of not being able to reach the distant metastases. If the toxicity to normal tissue could be overcome, combination of local and systemic injections would be more effective. Similarly, delivering low molecular weight agents (e.g.,

Table 1. Physiological promises and problems in the delivery of macromolecules to tumors

Promises

- Relatively high degree of specificity of antibodies for tumor-associated antigens
- Relatively large vascular permeability, interstitial diffusion coefficient, and hydraulic conductivity

Problems

- Heterogeneous blood supply
- Elevated interstitial pressure
- Fluid loss from periphery
- Large distances in the interstitium
- Large affinity and heterogeneous binding
- Metabolism

drug, toxin, enzyme, hormone) linked to MAbs and releasing them once they have extravasated or entered cells seems reasonable. However, once a small molecule is uncoupled from the antibody it may diffuse back into a nearby blood vessel, and may be rapidly eliminated.

Ideally, an antibody should have a high specificity and low molecular weight. To this end, recent developments in producing recombinant DNA monoclonal antibodies have already yielded smaller antibody fragments (e.g., antibody binding site, molecular recognition unit). In addition, two other approaches seem to satisfy the requirement of low molecular weight with increased specificity: the use of low molecular weight chelates with bifunctional antibodies (BFA) [54] and the use of low molecular weight prodrugs with enzyme-conjugated antibodies (ECA) [55]. In these two-step approaches, BFA (or ECA) is injected into a patient, permitted to bind to antigenic sites in the tumor, and then cleared from the normal tissues. At an appropriate time later, a radionuclide attached to a low molecular weight chelate (or a prodrug) is injected into the patient with the advantage of rapid delivery to the tumor and clearance from the body. An increase in antigen expression and/or affinity may help this approach. The number of antigenic sites may be increased using biological response modifiers such as interferon [56]. This would increase the concentration of antibody near the blood vessels, but would not increase the depth of penetration until the antigens are saturated [43, 44]. One way of overcoming some of the problems of poor antibody localization is to use radioisotopes with large tumor dose deposition and large depths of penetration; however toxicity to normal tissues may become a limiting factor. Protecting bone marrow using growth factors (e.g., interleukin-1, colony stimulating factors) or with bone marrow transplant may alleviate the normal tissue toxicity problem. Another method is to combine antibody treatment with other modalities (e.g., radiation sensitizers, low molecular weight cytotoxic drugs to synchronize cell cycle, hyperglycemia to lower pH [57]) depending upon the tumor type. Finally, antibodies directed against the necrotic tissue may ex-

ploit the 'reservoir' phenomenon seen in tumors [25-27, 43, 52].

In contrast, the physiological barriers discussed in this article may not be a problem for: (i) radioimmunodetection, (ii) treating leukemias, lymphomas and small tumors (e.g., micrometastases) in which the interstitial pressure is low and diffusion distances are small, (iii) treatment of adequately perfused, low pressure regions of large tumors [58], and (iv) treatment with antibodies directed against the tumor endothelial cells or microenvironment of the subendothelial matrix. We have recently shown that activation with lymphokines increases the rigidity of LAK cells [59]. These results suggested that the uptake of LAK cells might be increased by intra-arterial injection of these cells into an organ infiltrated by tumor metastases [59]. We have indeed found this to be true in VX2 carcinoma grown in rabbits [60]. These results suggest that in addition to the direct cytotoxicity against cancer cells, the LAK cell therapy may be vasculature mediated [60]. Thus LAK cells may be useful as drug carriers to the solid tumors [60, 61], and may complement anti-angiogenic therapeutic approaches [6]. In fact, the tumor vasculature and the subendothelial matrix may be a target for several conventional and novel therapies (e.g., TNF, hyperthermia, anti-angiogenic factors), and hence must be exploited in cancer treatment.

These physiological barriers may also not pose any problems for treatment with a molecule or cell which has nearly 100% specificity for cancer cells. Until such molecules or cells are developed, methods are urgently needed to overcome or exploit these physiological barriers in tumors. It is hoped that an improved understanding of tumor physiology will help in developing these strategies. In the meantime, the search for highly selective, tumor-specific agents must go on using *in vitro* methodologies. Once a novel therapeutic agent has been identified based on extensive *in vitro* screening, we need to ask whether this agent will arrive in all regions of a tumor, and more importantly, whether it will arrive there in therapeutically adequate quantities with minimal toxicity to normal tissues. Only after the answers to these questions is positive may the agent be considered as a breakthrough. It

is also hoped that the physiological insight presented in this article helps in answering these questions and subsequently becomes a prerequisite to the optimal development of novel therapeutic strategies for treatment of solid tumors including the development of anti-angiogenic modalities as well as the delivery of effector cells (e.g., lymphokine activated killer cells, tumor infiltrating lymphocytes) to the tumor microenvironment.

Key unanswered questions

- How different are the transport parameters of human tumors from those of animal tumors?
- How can the physiological barriers in tumors be overcome predictably and reproducibly using chemical or physical agents?
- What are the optimal size, charge, configuration and affinity of a molecule for cancer detection and treatment?
- How can the rigidity, adhesiveness and motility of activated lymphocytes be modulated to increase their localization in solid tumors?
- How can the physical and biological characteristics of the tumor vasculature and interstitium be exploited for cancer treatment?

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Notes

1. Boucher Y, Opacic D, Kirkwood JM, Jain RK: Elevated interstitial fluid pressure in human melanomas [Abstract], 16th Gray Conference - Vasculature as a Target for Anti-Cancer Therapy, Manchester, England, September 17-21, 1990

2. Roh HD, Boucher Y, Bloomer WD, Jain RK: Interstitial hypertension in human cervical carcinomas: Effect of radiation [Abstract], 16th Gray Conference - Vasculature as a Target for Anti-Cancer Therapy, Manchester, England, September 17-21, 1990

References

1. Jain RK: Delivery of novel therapeutic agents in tumors: Physiological barriers and strategies. *J Natl Cancer Inst* 81: 570-576, 1989
2. Gullino PM: The internal milieu of tumors. *Progr Exp Tumor Res* 8: 1-25, 1966
3. Jain RK, Weissbrod J, Wei J: Mass transfer in tumors: Characterization and applications in chemotherapy. *Adv Cancer Res* 33: 251-310, 1980
4. Gerlowski LE, Jain RK: Physiologically-based pharmacokinetics: Principles and applications. *J Pharm Sci* 72: 1103-1127, 1983
5. Gullino PM: Extracellular compartments of solid tumors. In: Cancer Becker FF (ed), pp 327-354. Plenum Publishing Corporation, New York 1975
6. Folkman J: Tumor angiogenesis. *Adv Cancer Res* 43: 175-203, 1985
7. Jain RK: Determinants of tumor blood flow: A review. *Cancer Res* 48: 2641-2658, 1988
8. Less J, Skalak T, Sevick EM, Jain RK: Microvascular architecture in a mammary carcinoma: Branching patterns and vessel dimensions. (Submitted)
9. Algire GH, Chalkley HW: Vascular reactions of normal and malignant tissues *in vivo*. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J Natl Cancer Inst* 6: 73-85, 1945
10. Gullino PM, Grantham FH: The vascular space of growing tumors. *Cancer Res* 24: 1727-1732, 1964
11. Jain RK, Ward-Hartley K: Tumor blood flow - characterization, modification, and role in hyperthermia. *IEEE Transactions on Sonics and Ultrasonics* SU-31: 504-526, 1984
12. Vaupel P, Kallinowski F, Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. *Cancer Res* 49: 6449-6459, 1989
13. Ide AG, Baker NH, Warren SL: Vascularization of the Brown-Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. *Am J Roentgenol* 41: 891-899, 1939
14. Endrich B, Reinhold HA, Gross JF, Intaglietta M: Tissue perfusion inhomogeneity during early tumor growth in rats. *J Natl Cancer Inst* 62: 387-395, 1979
15. Dudar TE, Jain RK: Differential responses of normal and tumor microcirculation to hyperthermia. *Cancer Res* 44: 605-612, 1984
16. Sevick EM, Jain RK: Geometric resistance to blood flow in

- solid tumors perfused *ex vivo*: Effects of tumor size and perfusion pressure. *Cancer Res* 49: 3506-3512, 1989
17. Sevick EM, Jain RK: Viscous resistance to blood flow in solid tumors: Effect of hematocrit on intratumor blood viscosity. *Cancer Res* 49: 3513-3519, 1989
 18. Straw J, Hart M, Klubes P, Zahrao D, Dedrick RL: Distribution of anticancer agents in spontaneous animal tumors. I. Regional blood flow and methotrexate distribution in canine lymphosarcoma. *J Natl Cancer Inst* 52: 1327-1331, 1974
 19. Beaney RP, Lammertsma AA, Jones T, McKenzie CG, Halnan KE: *In vivo* measurements of regional blood flow, oxygen utilization and blood volume in patients with carcinoma of the breast using positron tomography. *Lancet* 1: 131-133, 1984
 20. Jain RK: Transport of molecules across tumor vasculature. *Cancer and Metastasis Reviews* 6: 559-594, 1987
 21. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM: Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Amer J Pathol* 133: 95-109, 1988
 22. Sevick E, Jain RK: Measurement of capillary filtration coefficients in a solid tumor. *Microvascular Res*, in press
 23. Gerlowski LE, Jain RK: Effect of hyperthermia on microvascular permeability of normal and neoplastic tissues. *Intl J Microcirc: Clinical Exptl* 4: 336-372, 1985
 24. Jain RK: Transport of macromolecules in tumor microcirculation. *Biotech Progress* 1: 81-94, 1985
 25. Jain RK, Baxter LT: Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: Significance of elevated interstitial pressure. *Cancer Res* 48: 7022-7032, 1988
 26. Baxter LT, Jain RK: Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection. *Microvasc Res* 37: 77-104, 1989
 27. Baxter LT, Jain RK: Transport of fluid and macromolecules in tumors. II. Role of heterogeneous perfusion and lymphatics. *Microvasc Res*, in press
 28. Young JS, Lumsden CE, Stalker AL: The significance of the 'tissue pressure' of normal testicular and of neoplastic (Brown-Pearce carcinoma) tissue in the rabbit. *J Pathol Bacteriol* 62: 313-333, 1950
 29. Jain RK: Transport of molecules in the tumor interstitium: A review. *Cancer Res* 47: 3039-3051, 1987
 30. Boucher Y, Baxter LT, Jain RK: Interstitial fluid pressure gradients in tissue-isolated and subcutaneous tumors: Implications for therapy. *Cancer Res* 50: 4478-4487, 1990
 31. Wiig H, Tveit E, Hultborn R, Reed RK, Weiss L: Interstitial fluid pressure in DMBA-induced rat mammary tumors. *Scand J Clin Lab Invest* 42: 159-164, 1982
 32. Misiewicz M: Microvascular and interstitial pressures in normal and neoplastic tissues. Thesis MS, Carnegie Mellon University, 1986
 33. Sylven B, Bois I: I. Protein content and enzymatic assays of interstitial fluid from some normal tissues and transplanted mouse tumors. *Cancer Res* 20: 831-835, 1960
 34. Chary SR, Jain RK: Direct measurement of interstitial diffusion and convection of albumin in normal and neoplastic tissues using fluorescence photobleaching. *Proc Natl Acad Sci (USA)* 86: 5385-5389, 1989
 35. Swabb EA, Wei J, Gullino PM: Diffusion and convection in normal and neoplastic tissues. *Cancer Res* 34: 2814-2822, 1974
 36. Nugent LJ, Jain RK: Extravascular diffusion in normal and neoplastic tissues. *Cancer Res* 44: 238-244, 1984
 37. Gerlowski LE, Jain RK: Microvascular permeability of normal and neoplastic tissues. *Microvasc Res* 31: 288-305, 1986
 38. Clauss MA, Jain RK: Interstitial transport of rabbit and sheep antibodies in normal and neoplastic tissues. *Cancer Res* 50: 3487-3492, 1990
 39. Sutherland R, Buchegger F, Schreyer M, Vacca A, Mach JP: Penetration and binding of radiolabelled anti-carcinoembryonic antigen monoclonal antibodies and their antigen binding fragments in human colon multicellular tumor spheroids. *Cancer Res* 47: 1627-1633, 1987
 40. Astarita G: Mass Transfer with Chemical Reactions. Elsevier, Amsterdam, 1967
 41. Fujimori K, Covell DG, Fletcher JE, Weinstein JN: Modeling analysis of the global and microscopic distribution of immunoglobulin G, F(ab')₂, and Fab in tumors. *Cancer Res* 49: 5656-5663, 1989
 42. Dedrick RL, Flessner MF: Pharmacokinetic Considerations of Monoclonal Antibodies. In: Mitchell M (ed): Immunity to Cancer II, pp 429-438. Liss AR, New York, 1989
 43. Baxter LT, Jain RK: Transport of fluid and macromolecules in tumors. III. Role of binding and metabolism. (Submitted)
 44. Kaufman E, Jain RK: Simultaneous determination of transport and binding parameters using photobleaching: Potential for microcirculatory applications. *Biophys J*, in press
 45. Butler TP, Grantham FH, Gullino PM: Bulk transfer of fluid in the interstitial compartment of mammary tumors. *Cancer Res* 35: 3084-3088, 1975
 46. Vaupel P, Kallinowski F: Hemoconcentration of blood flowing through human tumor xenografts (Abstract). *Intl J Microcirc: Clin Exptl* 6: 72, 1987
 47. Sevick EM, Jain RK: Blood flow and venous pH of tissue-isolated Walker 256 carcinoma during hyperglycemia. *Cancer Res* 48: 1201-1207, 1988
 48. Jain RK: Tumor Blood Flow Response to Heat and Pharmacological Agents. In: Fielden EM, Fowler JF, Hendry JH, Scott D (eds) Radiation Research (Proc 8th ICRR) Vol 2, pp 813-818. London: Taylor and Francis, 1987
 49. Stickney DR, Grindley DS, Kirk GA, Slater JM: Enhancement of monoclonal antibody binding to melanoma with a single dose radiation or hyperthermia. *Natl Cancer Inst Monograph* 3: 47-52, 1985
 50. Mstrikale JS, Klein JL, Schroeder J, Order SE: Radiation enhancement of radiolabelled antibody deposition in tumors. *Intl J Radiat Oncol Biol Phys* 13: 1839-1844, 1987
 51. Neuwelt EA, Sprecht HD, Barnett RA, Dahlborg SA,

- Miley A, Larson SM, Brown P, McKernan KF, Hellstrom KE, Hellstrom I: Increased delivery of tumor specific monoclonal antibodies to brain after osmotic blood-brain barrier modification in patients with melanoma metastatic to central nervous system. *Neurosurgery* 20: 885-895, 1987
52. Jain RK, Wei I: Dynamics of drug transport in solid tumors: Distributed parameter model. *J Biomechanical Engineering* 1: 313-330, 1977
 53. Jain RK, Wei J, Gullino PM: Pharmacokinetics of methotrexate in solid tumors. *J Pharmacokin Biopharm* 7: 181-194, 1979
 54. Stickney DR, Slater JB, Kirk GA, Ahlem C, Chang C, Frincke JM: Bifunctional antibody: ZCE/CHA-indium-111-BLEDTA-(V) clinical imaging in colorectal carcinoma. *Antibody Immunocjugates and Radiopharmaceuticals* 2: 1-13, 1989
 55. Senter PD, Saulnier MG, Scheiber GJ, Hirschberg DL, Brown JP, Hellstrom I, Hellstrom KE: Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with cytoside phosphate. *Proc Natl Acad Sci USA* 85: 4847-4846, 1988
 56. Greiner JW, Quesadaui F, Noguchi P, Prestka S, Colcher D, Fisher PB, Schlom J: Recombinant interferon enhances monoclonal antibody-targeting of carcinoma lesions *in vivo*. *Science* 235: 895-898, 1987
 57. Ward KA, Jain RK: Physiological response of tumors to hyperglycemia: Characterization, significance, and role in hyperthermia. *International Journal of Hyperthermia* 4: 223-230, 1988
 58. Sittman JV, Order SE, Klein JJ, Leichner PK, Fishman EK, Smith GW: Conversion by new treatment modalities of nonresectable to resectable hepatocellular cancer. *J Clin Oncol* 5: 1566-1573, 1987
 59. Sasaki A, Jain RK, Maghazachi AA, Goldfarb RH, Herberman RB: Low deformability of LAK cells: A possible determinant of *in vivo* distribution. *Cancer Res* 49: 3742-3746, 1989
 60. Sasaki A, Melder RJ, Whiteside TL, Herberman RB, Jain RK: Preferential localization of human A-LAK cells in tumor microcirculation: A novel mechanism of adoptive immunotherapy (submitted)
 61. Basse P, Wasserman K, Herberman RB, Goldfarb RH: Selective accumulation of adoptively transferred A-LAK cells in metastases of mouse D16 melanoma (submitted)

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Constitutive and Antibody-induced Internalization of Prostate-specific Membrane Antigen¹

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Abstract

Prostate-specific membrane antigen (PSMA) is a cell surface glycoprotein expressed predominantly by prostate cancer cells. We have characterized four monoclonal antibodies that bind to the extracellular domain of PSMA (Liu et al., *Cancer Res.*, 57: 3629-3634, 1997). Here we report that viable LNCaP cells internalize these antibodies. Laser scanning confocal microscopy reveals that the internalized antibodies accumulate in endosomes, and immunoelectron microscopy reveals that endocytosis of the PSMA-antibody complex occurs via clathrin-coated pits. In addition, a quantitative cell surface biotinylation assay demonstrates that PSMA is constitutively endocytosed in LNCaP cells and that anti-PSMA antibodies increase the rate of internalization of PSMA. These studies suggest that PSMA might function as a receptor mediating the internalization of a putative ligand. The availability of prostate-specific internalizing antibodies should aid the development of novel therapeutic methods to target the delivery of toxins, drugs, or short-range isotopes specifically to the interior of prostate cancer cells.

Introduction

PSMA⁵ is the single most well-established highly restricted prostate epithelial cell membrane antigen (1-8). In contrast to other highly restricted prostate-related antigens such as prostate-specific antigen and prostatic acid phosphatase, which are secretory proteins, PSMA is an integral cell membrane protein. The PSMA gene has been cloned, sequenced (9), and mapped to chromosome 11q14 (10). One of the reasons for significant interest in PSMA is that it is ideal for *in vivo* prostate-specific targeting strategies. In addition to its prostate specificity (1-8), PSMA is expressed by a very high proportion of PCAs (1, 2, 4, 6, 7); expression is further increased in higher-grade cancers, metastatic disease (4, 6, 7), and hormone-refractory PCA (3, 6, 7). PSMA expression is modulated inversely by androgen levels (3, 6). Furthermore, PSMA expression has been found in tumor but not in normal vascular endothelium (7, 11), further broadening its interest and potential applications.

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⁵ The abbreviations used are: PSMA, prostate-specific membrane antigen; IEM, immunoelectron microscopy; IF, immunofluorescence; mAb, monoclonal antibody; PCA, prostate cancer.

PSMA has been shown to have peptidase (12) and folate hydrolase (13) activity. Although it shares some homology with rat brain *N*-acetylated α -linked acidic dipeptidase (12) and the transferrin receptor (9), PSMA does not share the latter's internalization signal (9). The function of PSMA with respect to PCA and vascular endothelial cell biology and the direct correlation between its expression and increasing PCA aggressiveness remain intriguing and unclear.

Until recently, the only available mAb to PSMA was 7E11.C5 (14), which targets an epitope located within the short cytoplasmic tail of the molecule (15, 16).⁶ As a result, 7E11.C5 did not bind viable cells (11, 14, 16). We recently reported the development of four IgG mAbs that react with the external domain and define two distinct epitopes of PSMA (PSMA_{ext1} and PSMA_{ext2}; Ref. 11). Because these mAbs are capable of binding viable PSMA-expressing cells, we have begun to use them in an effort to further understand the function of PSMA.

Materials and Methods

Antibodies and Reagents. mAbs J591, J415, and J533 (all IgG1) and E99 (IgG3) to PSMA_{ext1} and mAb I56 (IgG1; negative control) to inhibit were generated as described previously (9). Purified mAb 7E11.C5 was a generous gift from Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA). Secondary antibody reagents conjugated with FITC and Texas Red were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Antibody Uptake. LNCaP cells (15×10^4) were plated on glass coverslips in 35-mm dishes and grown for 2-3 days before initiating the experiments. For the internalization assay, the cells were washed in RPMI 1640 containing 0.5% fatty acid-free BSA (RPMI-BSA) and incubated with mAbs J591, J415, 7E11.C5, or I56 at 4 μ g/ml in RPMI-BSA at 37°C. When transferrin uptake was monitored, FITC-conjugated transferrin (Molecular Probes, Inc., Eugene, OR) was coincubated along with the respective antibody. The cells were washed and further processed for IF and confocal microscopy as described below. For immunoelectron microscopic detection of antibody uptake, the above-mentioned procedure was followed, except that the cells were grown directly on 35-mm culture dishes.

IF and Laser Scanning Confocal Microscopy. After primary mAb incubation, FITC-conjugated goat antimouse IgG [Jackson ImmunoResearch Laboratories: 1:100 in 1% BSA in PBS (pH 7.4)] was incubated for 30 min and washed extensively in 1% BSA in PBS. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

The internalization of FITC-conjugated transferrin and antibodies against PSMA was examined using a Phoibos 1000 laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA) as described previously (16). To detect FITC- and Texas Red-labeled reagents simultaneously, samples were excited at 514 nm with an argon laser; the light emitted between 525 and 540 nm was recorded for FITC, and the light emitted above 630 nm was recorded for Texas Red. Serial optical sections of the monolayer were recorded at 0.4 μ intervals. A total of 30-40 horizontal (X-Y) confocal sections were obtained for each cell type and used to generate three-dimensional images using the Image Space software program (version 3.01; Molecular Dynamics) on an Iris Indigo Workstation (Silicon Graphics, Mountain View, CA).

IEM. After antibody incubation at 37°C for 2 h, the cells were washed in PBS in BSA, fixed for 20 min in cold methanol, and hydrated in PBS in BSA. Cells were then incubated for 1 h with 15-nm gold beads conjugated with goat antimouse IgG (Amersham Life Science, Inc., Arlington Heights, IL). After washing, the cells were fixed in 2.5% glutaraldehyde for 15 min, scraped gently, pelleted, and processed for IEM as described previously (11, 17). Electron micrographs were taken with a Joel 100 CX electron microscope.

Cell Surface Biotinylation Assay for Endocytosis. Biotinylation assays were performed as described by Bretscher and Lutter (18). Briefly, LNCaP cells (60×10^4) were grown on polylysine (3%) coated 60-mm dishes. Cells were washed in precooled PBS containing 1 mM each of calcium chloride and magnesium chloride. To biotinylate the cell surface proteins, cells were treated with the water-soluble, membrane-impermeable, cleavable biotin analogue sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-biotin; Pierce Chemical Co., Rockford, IL; 0.5 mg/ml) at 4°C for 20 min and then washed in RPMI-BSA. Two control dishes were kept on ice, whereas the other dishes were incubated at 37°C. The incubation was stopped at various times by transferring cells back to 4°C. After washing in 10% FCS in PBS, the cells were incubated twice for 20 min in reducing solution [310 mg of glutathione-free acid (Sigma, St. Louis, MO) dissolved in 17 ml of H₂O; 1 ml of 1.5 M NaCl, 0.12 ml of 50% NaOH, and 2 ml of serum were added just before use] to remove the residual cell surface exposed biotin. One control dish was reduced, and the second dish was not reduced, thereby serving as 0 and 100% biotinylation references, respectively. After washing, free sulfhydryl groups were quenched in iodoacetamide (5 mg/ml; Sigma) in BSA in PBS for 15 min. Cells were lysed, and PSMA was immunoprecipitated as described previously (11). Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions. The gels were transferred to nitrocellulose membranes, and the blots were probed with ¹²⁵I-streptavidin (Amersham), autoradiographed, and quantified using a densitometer (Molecular Dynamics).

Results

Staining of Viable Cells and Internalization of mAbs. IF analysis of viable LNCaP cells incubated with mAbs J591, J533, E99, and J415 at 4°C showed distinct plasma membrane staining (data not shown), whereas mAb 7E11.C5 revealed no plasma membrane staining. Incubation of cells at 37°C with mAb J591 revealed labeling of both plasma membrane and intracellular vesicles (Fig. 1). After a 5-min incubation at 37°C, the labeling was detected primarily on the plasma membrane (Fig. 1A). At 20 min, distinct staining of intracellular vesicles was apparent (Fig. 1B), and at 180 min, intense labeling was observed in the juxtanuclear region, with sparse labeling throughout the cytoplasm (Fig. 1C). mAbs J415, J533, and E99 gave identical results (data not shown). mAb 7E11.C5 showed neither cell surface nor intracellular staining (data not shown) in these viable cells. These results indicate that mAbs to PSMA_{ext} are internalized by viable PCA cells.

Endosomal Localization of Internalized Antibodies. To test whether internalized antibodies accumulate in endosomes, a simultaneous uptake of mAbs and FITC-labeled transferrin (an endosomal marker) was carried out. Laser scanning confocal microscopy revealed that internalized J591 (Fig. 2A) and transferrin (Fig. 2C) codistributed to a large extent (Fig. 2E), indicating that the internalized PSMA-antibody complex accumulates in endosomes. Control experiments with 7E11.C5 confirmed that this antibody is not internalized (Fig. 2, B, D, and F).

IEM. IEM of nonpermeabilized LNCaP cells at 4°C revealed mAb J591 binding to the extracellular side of the plasma membrane (11). IEM of viable LNCaP cells incubated with mAb J591 at 37°C for 10 min showed an accumulation of gold particles in clathrin-coated pits (Fig. 3, A and B) and in vesicles close to the plasma membrane (Fig. 3C). After a 2-h incubation at 37°C, vesicles containing gold beads were found in a juxtanuclear location (Fig. 3D). These findings indicate that mAb J591 internalization occurs via clathrin-coated pits in LNCaP cells.

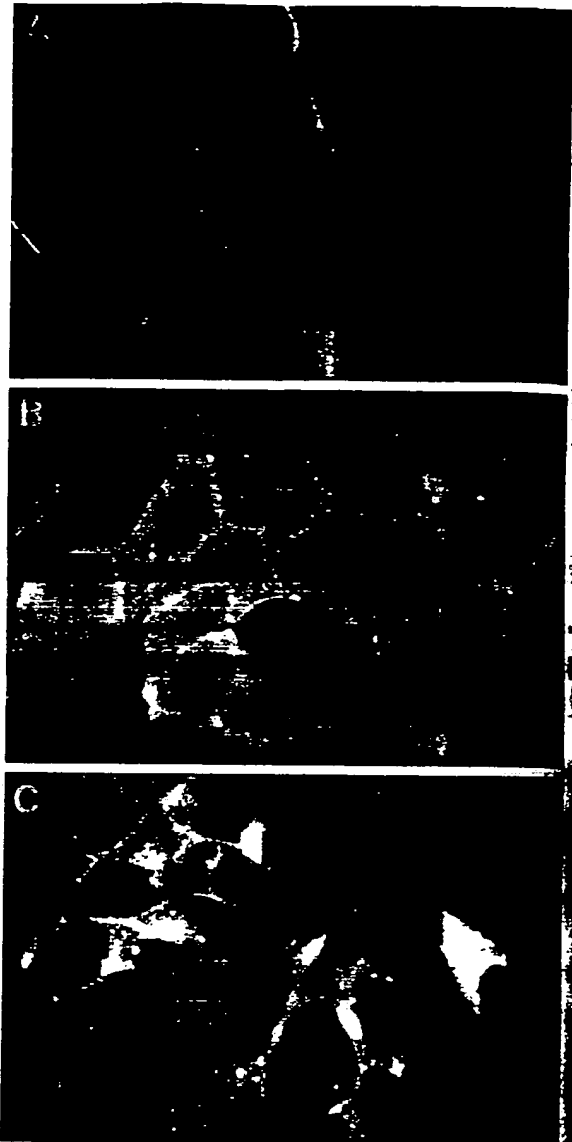
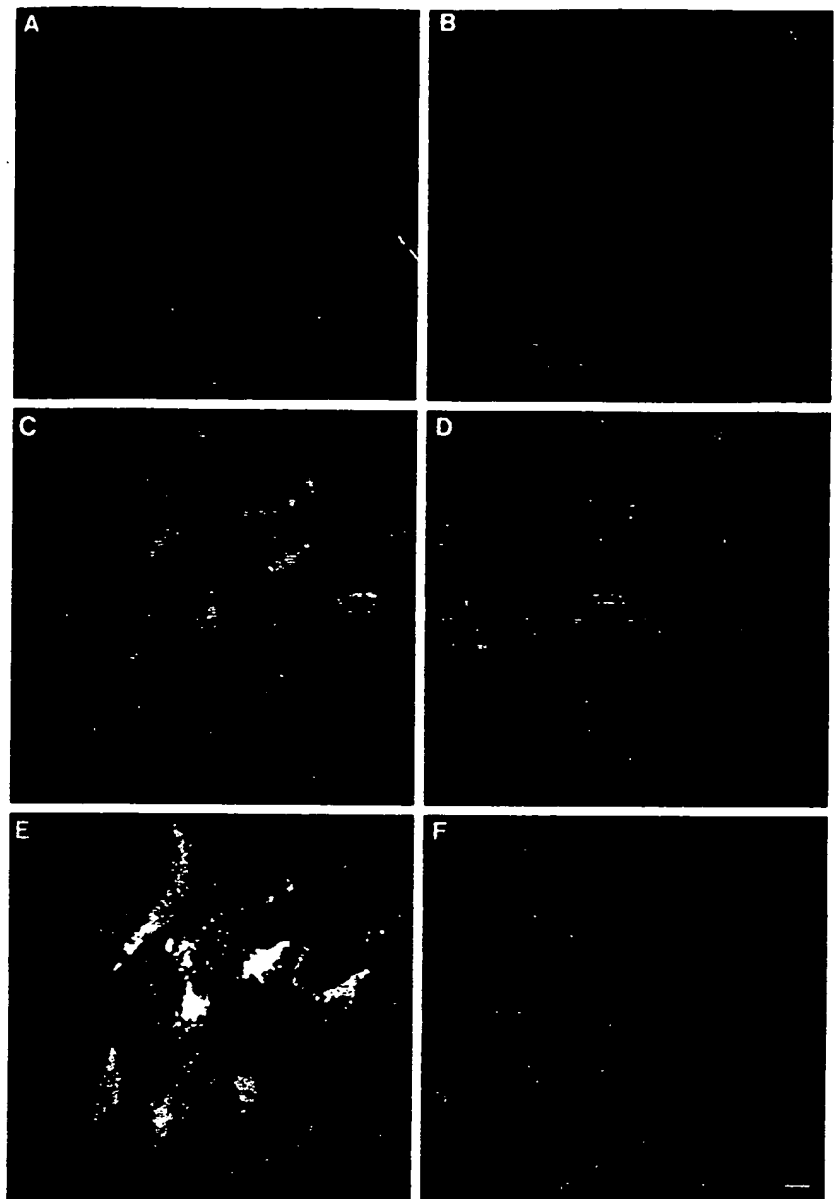


Fig. 1. Internalization of mAb J591 in LNCaP cells. Live cells were incubated with mAb J591 for 5 (A), 20 (B), and 180 (C) min. Cells were then permeabilized and stained with FITC-conjugated secondary antibody to visualize internalized mAb J591.

Internalization of PSMA. A cell surface protein biotinylation assay was developed to test whether PSMA is internalized in the absence of antibody, or whether antibody binding induces PSMA internalization. In this assay, a cleavable biotin analogue (NHS-SS-biotin) was used to label proteins exposed on the surface at 4°C. The return of surface biotinylated cells to a temperature of 37°C allows the internalization of the appropriate cell surface proteins with their biotin tag. The NHS-SS-biotin label is removed from noninternalized cell surface proteins through cleavage of the disulfide linkage with glutathione (18), whereas internalized biotinylated proteins are protected from this cleavage. The appearance of biotin-labeled protein that is resistant to glutathione reduction was taken as an indicator of internalization.

Fig. 2. Confocal microscope analysis of the internalization of mAb J591. LNCaP cells were incubated with mAb J591 and FITC-conjugated transferrin (A, C, and E) or mAb 7E11.C5 and FITC-conjugated transferrin (B, D, and F) for 2 h and processed for IF as described in "Materials and Methods." mAbs J591 (A) and 7E11.C5 (B) were detected with a Texas Red-conjugated secondary antibody. FITC-conjugated transferrin uptake is shown (C and D). Images in A and C were merged to obtain the image in E (mAb J591 and FITC-conjugated transferrin colocalization, yellow). Images in B and D were merged to obtain the image in F, in which only transferrin uptake is seen because 7E11.C5, neither binds nor internalizes.



nalization. As shown in Fig. 4A, biotinylated PSMA is sensitive to glutathione reduction after labeling cells at 4°C (Fig. 4A, compare Lanes 1 and 2). However, with progressively longer incubation periods at 37°C, an increasing proportion of the labeled PSMA becomes increasingly resistant to reduction (Fig. 4A, Lanes 3–6). Quantitation of the blots revealed that 60% of the total cell surface PSMA was internalized (Fig. 4C) within 2 h.

When the biotinylated cells were incubated with 1 µg/ml mAb J591 at 37°C, a 3-fold increase in the rate of internalization of PSMA was observed (compare Fig. 4B, Lanes 3 and 4 and Fig. 4D). A higher concentration of mAb J591 did not show any further increase in the internalization of PSMA (Fig. 4B, Lanes 5–7; Fig. 4D). Similar results were obtained with monovalent Fab fragments (data not shown).

Discussion

The prostate-restricted nature of PSMA, coupled with the direct association between the level of PSMA expression and increasingly aggressive disease (4), implies a potentially important role for PSMA in PCA biology. The importance of understanding the function of PSMA is further stimulated by its expression in vascular endothelium specifically supplying cancers but not in normal, resting endothelium (7, 11). In the past, investigating PSMA function has been compromised because the sole antibody to PSMA reacted with a cytoplasmic epitope of the molecule and therefore bound only to cells that were permeabilized or dead (11, 14, 16). Our recent development of mAbs to the extracellular domain of PSMA and their demonstrated ability to

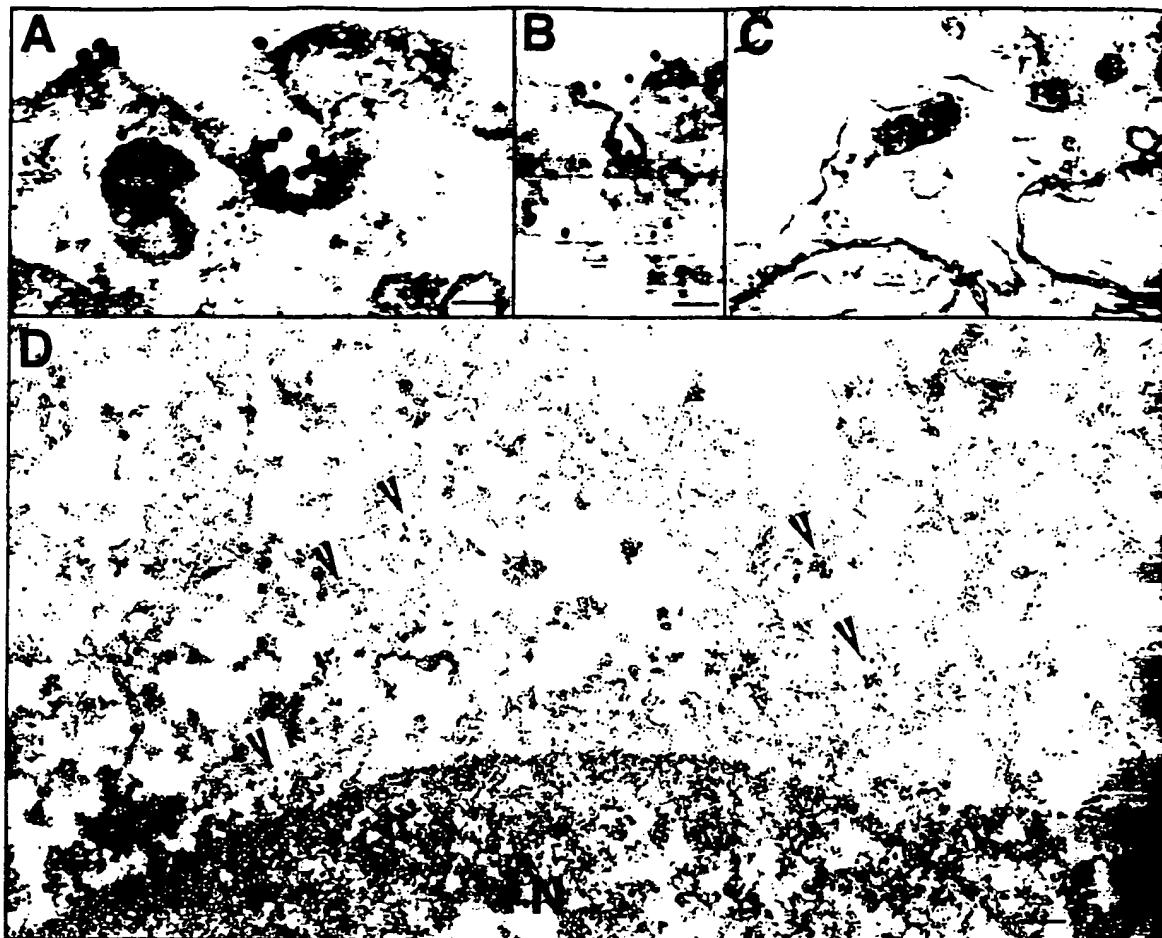


Fig. 3. IEM of the internalized mAb J591 in LNCaP cells. Cells were incubated with J591 at 37°C for 10 min (A–C) or 2 h (D) and processed for immunogold labeling as described in "Materials and Methods." Note the accumulation of gold particles in clathrin-coated vesicles (A and B) and in vesicles proximal to the plasma membrane (C). At 2 h, note the accumulation of gold particles in a juxtanuclear region (arrowheads). N, nucleus. Bars represent 34 (A), 65 (B and C), and 85 nm (D), respectively.

bind viable cells (11) have provided a means to study the function of PSMA.

In this study, we demonstrate by a combination of microscopical and biochemical techniques that PSMA and mAbs to PSMA_{ext} are internalized by LNCaP cells. Confocal microscopy and IEM reveal that PSMA-mAb complexes are endocytosed via clathrin-coated pits (Figs. 2 and 3). A quantitative cell surface biotinylation assay demonstrates that PSMA is constitutively internalized in the absence of antibody binding. At 20 min, 15% of the total biotinylated surface PSMA is internalized (Fig. 4C). The proportion of surface PSMA internalized increases to 60% at 60 min and remains fairly constant at that level for 240 min thereafter, when the assay was terminated. The stability of the labeled PSMA for a period of over 6 h (data not shown) indicates that PSMA degradation during this period is minimal. Internalization of only 60% of the total labeled surface PSMA may be explained by the recycling of internalized, biotinylated PSMA back to the cell surface,⁶ where it would be reduced and rendered undetectable in this assay.

⁶ H. Liu, R. Rahmati, and N. H. Bander, unpublished observations.

Constitutive internalization of PSMA may reflect the recycling of structural protein through a plasma membrane location or may be mediated by the binding of a ligand. Whereas the finding that PSMA antibody significantly increases the rate of internalization of PSMA is consistent with the latter ligand receptor-type function, it does not necessarily indicate that PSMA has a transport function. In the presence of mAb to PSMA_{ext}, the rate of internalization of PSMA increased up to 3-fold in a dose-dependent manner, reaching a maximum rate at an antibody concentration of 1–2 µg/ml (Fig. 4). A similar increase in the internalization rate has been shown for epidermal growth factor and its ligand (19).

It is well established that many ligands and their transmembrane receptors are internalized via clathrin-coated pits (receptor-mediated endocytosis) (20). The formation of antigen-antibody complexes on the cell surface often results in internalization through a pathway closely resembling the receptor-mediated endocytosis of peptide hormones, growth factors, and other natural ligands (21). Based on our findings, we hypothesize that PSMA may have a transport function as an as yet unidentified ligand. The baseline internalization rates of PSMA may indicate that PSMA may internalize in the absence

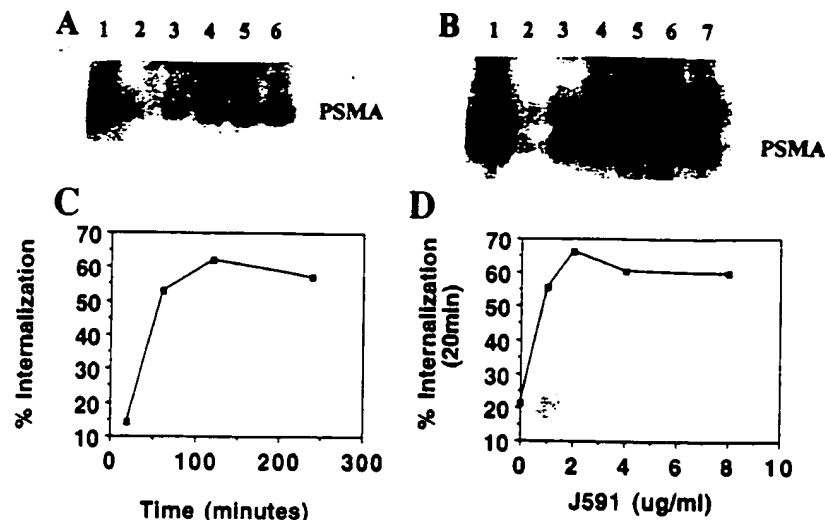


Fig. 4. mAb-independent (A and C) and mAb-induced (B and D) internalization of PSMA. The cell surface was biotinylated at 4°C with NHS-SS-biotin and then transferred to 37°C. Biotin was reduced at 4°C with glutathione as described in "Materials and Methods." PSMA was immunoprecipitated, run on a 10% SDS-PAGE, and detected with 125 I-streptavidin. Lanes in A are as follows: Lane 1, incubation at 40°C with no reduction (100% control); Lane 2, incubation at 4°C followed by reduction (0% control); and Lanes 3–6, incubation at 37°C for 20, 60, 120, and 240 min followed by reduction. Note the complete reduction of biotinylated PSMA in Lane 2 compared with Lanes 3–6, indicating that PSMA is internalized. Densitometric quantitation (C) revealed that approximately 60% of the total cell surface PSMA is internalized. mAb J591 induced the internalization of PSMA (B and D). LNCaP cells were biotinylated at 4°C and incubated with different amounts of mAb J591 for 20 min at 37°C. Lanes in B are as follows: Lane 1, incubation at 4°C with no reduction; Lane 2, incubation at 4°C followed by reduction; and Lanes 3–7, incubation at 37°C with 0, 1, 2, 4, and 8 µg/ml J591 followed by reduction. Note the increased uptake of PSMA in the presence of 1 µg/ml J591 (Lane 4) compared with Lane 3, in which no antibody is present. Increasing the mAb J591 concentration above 1 µg/ml did not increase the uptake, indicating a saturation of PSMA uptake. Densitometric quantitation (D) revealed that 1 µg/ml mAb J591 increased the uptake of PSMA by approximately 3-fold.

ligand, or, alternatively, that the PSMA ligand may be present in the culture medium. Similarly, mAb or mAb fragments act as a surrogate ligand, inducing an increased rate of internalization. The internalization pattern seen in this study may have been influenced or modified by the presence of mAb (22) and may not reflect the natural internalization pattern.

The targeting of most receptors to coated pits and their traffic through the endocytic compartment are thought to be mediated by a specific internalization motif in the cytoplasmic domain of the receptor (20). The first well-characterized internalization motifs of several receptors, including the transferrin receptor, mannose-6-phosphate receptor, asialoglycoprotein receptor, polymeric immunoglobulin receptor, and others, are all tetrapeptides (Tyr-X-Arg-Phe) having an aromatic residue in the fourth position of the sequence (23). The cytoplasmic tail of PSMA lacks a sequence similar to the Tyr-X-Arg-Phe motif (9). Another signal is the dileucine motif, for which the only known requirement is the presence of two consecutive leucines or a leucine-isoleucine pair. The dileucine motif has been shown to mediate internalization and targeting to endosomes and lysosomes (24). A dileucine motif is present in the cytoplasmic tail of PSMA. Experiments are under way to confirm the dileucine internalization motif of PSMA. Interestingly, whereas PSMA is 85% homologous to a rat brain neuropeptidase (24), this homology is located primarily at the COOH-termini and declines to less than 50% homology at the NH₂-termini. Furthermore, rat brain neuropeptidase lacks both the Tyr-X-Arg-Phe and dileucine motifs (25) and presumably does not internalize. Therefore, the highly restricted expression of PSMA becomes increasingly PCA-specific via different mechanisms. For example, at the mRNA level, normal and benign hyperplastic prostate epithelia predominantly express the cytosolic PSM' splice variant without a significant membrane-expressed component, whereas in PCA, the membrane form predominates by 10–100-fold

(26). Another form of functional specificity is demonstrated in rat brain astrocytes (25); although there is expression of a homologous neuropeptidase, this neuropeptidase is presumably not internalized as is PSMA in PCA cells.

The property of mAbs to PSMA_{ext} to be internalized in PCA cells adds another dimension to their *in vivo* therapeutic potential. In addition to selective/specific binding to the PCA cell surface, the mAb or fragment would be internalized into the targeted cells, providing direct access to the neoplastic cell machinery. As such, this property opens up options such as the use of toxin or drug conjugates. Similarly, the juxtanuclear location of the internalized vesicles should increase the potency of mAb- α particle conjugates by improving the incident angle of the isotope and the target DNA.

Lastly, although mAb may function as a surrogate ligand, the question remains as to the identity of the putative natural ligand of PSMA. Troyer *et al.* (16) noted a *M*_r 40,000 band that coimmunoprecipitated with PSMA that they identified as *S*-glutamic oxalacetic transaminase. We have not been able to demonstrate the binding of *S*-glutamic oxalacetic transaminase to PSMA (data not shown). Further study will be required to define the putative natural ligand, which, in turn, may shed additional light on the role of PSMA in cancer biology and tumor angiogenesis. The natural ligand, if similarly restricted in its tissue receptor binding profile, may substitute for the mAb in a targeted therapy approach.

Acknowledgments

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References

- Zhang, S., Zhang, H. S., Reuter, V. E., Slovin, S. F., Scher, H. I., and Livingston, P. O. Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. *Clin. Cancer Res.* 4: 295–302, 1998.

2. Lopes, D., Davis, W. L., Rosenstrauss, M. J., Uveges, A. J., and Gilman, S. C. Immunohistochemical and pharmacokinetic characterization of the site-specific immunocytotoxic conjugate CYT-356 derived from anti-prostate monoclonal antibody 7E11-C5. *Cancer Res.*, 50: 6423-6429, 1990.
3. Israeli, R. S., Powell, C. T., Corr, J. G., Fair, W. R., and Heston, W. D. W. Expression of the prostate-specific membrane antigen. *Cancer Res.*, 54: 1807-1811, 1994.
4. Wright, G. L., Jr., Haley, C., Beckett, M. L., and Schellhammer, P. F. Expression of prostate-specific membrane antigen in normal, benign, and malignant prostate tissues. *Urol. Oncol.*, 1: 18-28, 1995.
5. Troyer, J. K., Beckett, M. L., and Wright, G. L., Jr. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int. J. Cancer*, 62: 552-558, 1995.
6. Wright, G. L., Jr., Grob, B. M., Haley, C., Grossman, K., Newhall, K., and Moriarty, R. Up-regulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology*, 48: 326-334, 1996.
7. Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D. W., and Cordon-Cardo, C. Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res.*, 3: 81-85, 1997.
8. Sukoloff, R. L., Norton, K. C., Gasior, C. L., Marker, K. M., and Grauer, L. S. Quantification of prostate specific membrane antigen (PSMA) in human tissues and subcellular fractions. *Proc. Am. Assoc. Cancer Res.*, 39: 265, 1998.
9. Israeli, R. S., Powell, C. T., Fair, W. R., and Heston, W. D. W. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.*, 53: 227-230, 1993.
10. Rinker-Schaeffer, C. W., Hawkins, A. L., Su, S. L., Israeli, R. S., Griffin, C. A., Isaacs, J. T., and Heston, W. D. W. Localization and physical mapping of the prostate-specific membrane antigen (PSM) gene to human chromosome 11. *Genomics*, 30: 105-108, 1995.
11. Liu, H., Moy, P., Kim, S., Xia, Y., Rajasekaran, A., Navarro, V., Knudson, B., and Bander, N. H. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor endothelium. *Cancer Res.*, 57: 3629-3634, 1997.
12. Carter, R. E., Feldman, A. R., and Coyle, J. T. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc. Natl. Acad. Sci. USA*, 93: 749-753, 1996.
13. Pinto, J. T., Suffoletto, B. P., Berzin, T. M., Qiao, C. H., Lin, S., Tong, W. P., May, F., Mukherjee, B., and Heston, W. D. W. Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.*, 2: 1445-1451, 1996.
14. Horoszewicz, J. S., Kawinski, E., and Murphy, G. P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. *Anticancer Res.*, 7: 927-936, 1987.
15. Troyer, J. K., Feng, Q., Beckett, M. L., and Wright, G. L., Jr. Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate-specific membrane antigen. *Urol. Oncol.*, 1: 29-37, 1995.
16. Troyer, J. K., Beckett, M. L., and Wright, G. L., Jr. Location of prostate-specific membrane antigen in the LNCaP prostate carcinoma cell line. *Prostate*, 30: 232-242, 1997.
17. Rajasekaran, A. K., Hojo, M., Huima, T., and Rodriguez-Boulon, E. Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. *J. Cell Biol.*, 132: 451-463, 1996.
18. Bretscher, M. S., and Lutter, R. A new method for detecting endocytosed proteins. *EMBO J.*, 7: 4087-4092, 1988.
19. Haigler, H. T., McKenna, J. A., and Cohen, S. Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor. *J. Cell Biol.*, 83: 82-90, 1979.
20. Mukherjee, S., Ghosh, R. N., and Maxfield, F. R. Endocytosis. *Physiol. Rev.*, 77: 759-803, 1997.
21. Pastan, I. H., and Willingham, M. C. Receptor-mediated endocytosis of hormones in cultured cells. *Annu. Rev. Physiol.*, 43: 239-250, 1981.
22. Milhiet, P. C., Giocondi, M.-C., Crine, P., Le Grimellec, C., Roques, B. P., and Garbay-Jaureguiberry, C. Fate of a fluorescent inhibitor of endopeptidase-24.11 using enzyme-expressing MDCK cells. Modification of its cellular processing with a monoclonal antibody. *Eur. J. Cell Biol.*, 65: 258-268, 1994.
23. Trowbridge, I. S. Endocytosis and signals for internalization. *Curr. Opin. Cell Biol.*, 3: 634-641, 1991.
24. Letourneur, F., and Klausner, R. D. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell*, 69: 1143-1157, 1992.
25. Luthi-Carter, R., Berger, U. V., Barczak, A. K., Enna, M., and Coyle, J. T. Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. *Proc. Natl. Acad. Sci. USA*, 95: 3215-3220, 1998.
26. Su, S. L., Huang, I.-P., Fair, W. R., Powell, C. T., and Heston, W. D. W. Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. *Cancer Res.*, 55: 1441-1443, 1995.

Monoclonal Antibodies to the Extracellular Domain of Prostate-specific Membrane Antigen Also React with Tumor Vascular Endothelium¹

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Abstract

Prostate-specific membrane antigen (PSMA), initially defined by monoclonal antibody (mAb) 7E11, is a now well-characterized type 2 integral membrane glycoprotein expressed in a highly restricted manner by prostate epithelial cells. 7E11 has been shown to bind an intracellular epitope of PSMA that, in viable cells, is not available for binding. Herein, we report the initial characterization of the first four reported IgG mAbs that bind the external domain of PSMA. Competitive binding studies indicate these antibodies define two distinct, noncompeting epitopes on the extracellular domain of PSMA. In contrast to 7E11, these mAbs bind to viable LNCaP cells *in vitro*. In addition, they show strong immunohistochemical reactivity to tissue sections of prostate epithelia, including prostate cancer. These mAbs were also strongly reactive with vascular endothelium within a wide variety of carcinomas (including lung, colon, breast, and others) but not with normal vascular endothelium. These antibodies should prove useful for *in vivo* targeting to prostate cancer, as well as to the vascular compartment of a wide variety of carcinomas.

Introduction

PSMA³ is a highly restricted prostate epithelial cell membrane glycoprotein of approximately 100 kDa (1, 2). The PSMA gene has been cloned, sequenced (2), and mapped to chromosome 11q14 (3). In contrast to other highly restricted prostate-related antigens such as PSA, prostatic acid phosphatase, and PSP, which are secretory proteins, PSMA is an integral membrane protein. Among the reasons for significant interest in PSMA is that it is ideal for *in vivo* prostate-specific targeting strategies. In addition to its prostate specificity (1, 2, 4, 5), PSMA is expressed by a very high proportion of PCas (6); this expression is further increased in higher-grade cancers, in metastatic disease (6), and in hormone-refractory PCa (5-7).

The initial validation of PSMA as an *in vivo* target has been borne out by imaging trials with mAb 7E11/CYT-356 (8-11). However, epitope mapping indicates that 7E11/CYT-356 targets an intracellular epitope (12, 13). In viable cells, this binding site is not accessible to

an antibody (1, 13). Successful imaging with 7E11/CYT-356 probably relates to the targeting of dead/dying cells within tumor sites (6, 12, 13). It has been noted (2, 12-14) that a mAb to the extracellular domain would provide benefits, including improved *in vivo* localization and enhanced imaging and therapy. In this study, we report the development of four IgG mAbs to the external domain of PSMA. These mAbs also have been found reactive to vascular endothelium within a wide range of carcinomas but not with normal endothelial cells.

Materials and Methods

Generation and Production of mAb. BALB/c mice were immunized three times with LNCaP cells or a primary culture of PCa epithelial cells. Spleen cells were fused with X63.Ag.653 mouse myeloma cells using standard hybridoma technique. Clones that were reactive against LNCaP but unreactive against tissue sections of normal kidney (with the exception of some proximal tubule reactivity) and colon were subcloned. Murine ascites fluid was produced, and mAbs were purified using protein G (Pharmacia LKB Biotechnology, Piscataway, NJ). Purified mAb 7E11 was a generous gift from Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA).

Immunohistochemical Staining. Normal and cancer tissues were pre-cooled in liquid nitrogen, snap-frozen in OCT compound (Miles Inc., Elkhart, IN) on dry ice, and stored at -80°C. Cryostat tissue sections (5 µm) were fixed in cold acetone (4°C) for 10 min. mAbs (5 µg/ml or hybridoma supernatants) were incubated for 1 h at RT. Antibody binding was detected using rabbit antimouse immunoglobulin-peroxidase (DAKO Corp., Carpinteria, CA) as a secondary antibody and diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as chromogen. Isotype-matched irrelevant antibody was used as a negative control.

Cross Immunoprecipitation. LNCaP cells were lysed in lysis buffer [20 mM Tris/HCl, pH 8; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; and 1% (v/v) Triton X-100]. The resulting lysate was precleared by incubation with protein G beads overnight at 4°C, then incubated with mAb for 2 h. Protein G beads were added for 90 min prior to further washing. The beads were resuspended and boiled for 5 min in 1× Laemmli sample buffer containing 2-mercaptoethanol at 5% final concentration. The samples were centrifuged, and supernatant was recovered and placed on a 12% SDS-PAGE gel. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5% dry milk/TBST (Tris-buffered saline-Tween 20), incubated with primary mAbs × 60 min and followed by sheep antimouse immunoglobulin-peroxidase (Amersham Corp., Cleveland, OH). After washing, the membranes were developed using the enhanced chemiluminescence method (Amersham). Either 0.15 µg/ml J591 or 0.5 µg/ml 7E11 was used as a probe to detect the protein that was immunoprecipitated by 7E11, J591, J533, J415, and E99, respectively. Isotype-matched irrelevant antibody (I56; reactive to PSP) was used as a negative control.

IF Assay. LNCaP cells were grown on glass coverslips. IF assays were performed using either viable or fixed cells, the latter being either permeabilized or nonpermeabilized. For fixation, cells were treated with 2% paraformaldehyde-PBS (PBS, pH 7.4) for 30 min at RT, which does not permeabilize the cell membrane, washed with 1% BSA-PBS, quenched for 10 min in 50 mM NH₄Cl in PBS, and rinsed with 1% BSA-PBS. Where cell membrane perme-

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³ The abbreviations used are: PSMA, prostate-specific membrane antigen; PSA, prostate-specific antigen; IEM, immunoelectron microscopy; IF, immunofluorescence; mAb, monoclonal antibody; PCa, prostate cancer; PSP, prostate secretory protein; RT, room temperature.

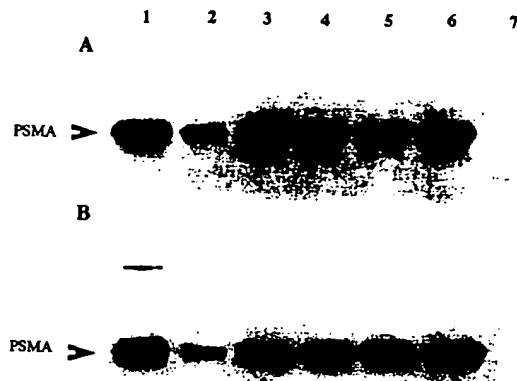


Fig. 1. Cross immunoprecipitation shows mAbs J591, J415, J533, E99, and 7E11 recognize the same antigen. Lane 1, crude LNCaP lysate. In Lanes 2-7, LNCaP lysate was immunoprecipitated with 5 μ g/ml mAb 7E11 (Lane 2); 5 μ g/ml J591 (Lane 3); 10 μ g/ml J533 (Lane 4); 10 μ g/ml J415 (Lane 5); 10 μ g/ml E99 (Lane 6); and 10 μ g/ml J56 (to PSP; Lane 7). Immunoprecipitates were immunoblotted with 0.15 μ g/ml mAb J591, (A) or 0.5 μ g/ml 7E11 (B).

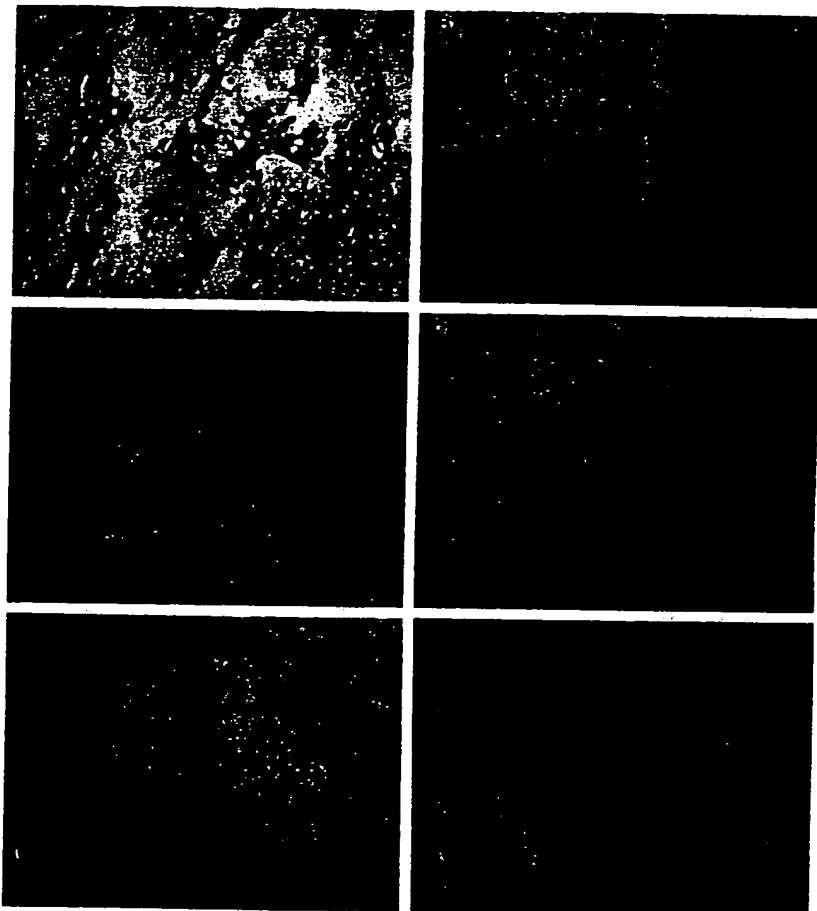
abilization was desired, 0.075% saponin (Sigma) in 1% BSA-PBS was added for 15 min at RT.

Primary mAb at 4 μ g/ml in BSA-PBS (plus saponin in cases of permeabilization) was incubated for 60 min at 4°C in the case of viable cells or at RT for fixed cells. After primary mAb incubation, viable cells were fixed in cold methanol for 20 min. FITC-goat antimouse secondary antibody (1:100 in BSA:PBS \pm saponin; Jackson ImmunoResearch, West Grove, PA) was incubated for 30 min and washed extensively in 1% BSA-PBS. Slides were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA).

IEM Microscopy. The IEM procedure was similar to the nonpermeabilized IF assay above. LNCaP cells were grown in 35-mm culture dishes and incubated with 10 μ g/ml J591 or 10 μ g/ml 7E11 for 45 min at 4°C, fixed with 2% paraformaldehyde, washed, and quenched as above. After 1% BSA washes, cells were incubated with 15-nm gold-conjugated goat antimouse IgG (Amersham) for 1 h. After extensive washing, cells were fixed in 2.5% glutaraldehyde for 15 min, gently scraped, pelleted, and processed for IEM as described previously (15). Electron micrographs were taken with a Joel 100CX electron microscope.

Competitive Binding Assay. Biotinylated mAbs were prepared by incubating 1 mg/ml mAb with 0.1 ml of biotinamidocaproate *N*-hydroxysuccinamide ester (1 mg/ml; Sigma) in DMSO for 4 h at RT. Unbound biotin was removed by dialysis against PBS. 7E11 (10 μ g/ml) was coated onto Terasaki plates. Plates were washed with 1%BSA-PBS-0.1% Tween 20. LNCaP membrane preparations were added to wells for 1 h at RT. After washing, serial dilutions of unlabeled (competing) antibody were added to duplicate wells for 1 h. Biotinylated antibody was added to each well and incubated for an additional 1 h followed by avidin-alkaline phosphatase (Sigma). After wash-

Fig. 2. Immunohistochemical reactivity of mAb J591 to neovasculature of renal (A), urothelial (B), colon (C), lung (squamous; D), breast carcinomas (E), and metastatic adenocarcinoma to liver. (F). $\times 250$.



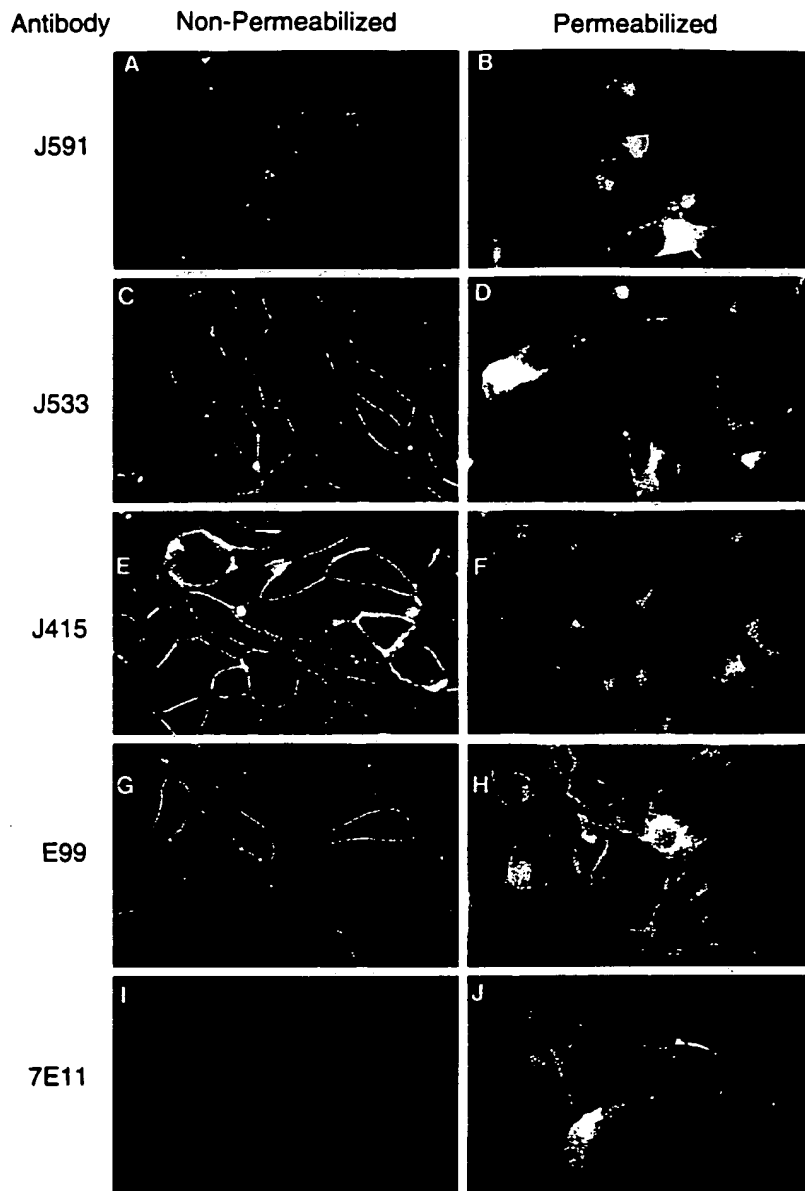


Fig. 3. Immunofluorescence assay comparing the reactivity of mAbs J591, J415, J533, and E99 to 7E11 on nonpermeabilized and permeabilized LNCaP cells. Intact, nonpermeabilized cells are reactive with mAbs J591 (A), J533 (C), J415 (E), and E99 (G) but not 7E11 (I). Reactivity is limited to the cell membrane without cytoplasmic staining, as mAbs do not enter the intact cells. Failure of 7E11 to bind (I) is consistent with the intracellular location of its epitope. When the cells are permeabilized prior to mAb incubation (B, D, F, H, J), reactivity to both cytoplasmic and membrane PSMA is seen. After the permeabilization and exposure of the intracellular PSMA epitope, 7E11 does bind. $\times 1500$.

ing, substrate (para nitrophenylphosphate) was added, and reactivity was read at $A_{405\text{ nm}}$. Irrelevant antibody (I56) was used as a control.

Results

From over 2000 clones screened, 4 clones that reacted with a 100-kDa band on Western blots and that produced strong immunohistochemical staining of prostate epithelium were selected for further characterization.

Immunoprecipitation/Immunoblot. In Western blot analysis, mAbs J591 (IgG1), J533 (IgG1), J415 (IgG1), and E99 (IgG3), as well as 7E11, identified a 100-kDa band from LNCaP lysate but not from the PSMA-negative PC3 lysate (data not shown). To confirm that mAbs J591, J533, J415, and E99 detected the same antigen as 7E11,

a cross-immunoprecipitation experiment was performed. Fig. 1 illustrates that the 100-kDa band that was immunoprecipitated by mAbs J591, J533, J415, E99, or 7E11 was detectable by immunoblot using either J591 or 7E11 as a probe (Fig. 1, A and B, respectively). Sequential immunoprecipitation studies (data not shown) also demonstrated that 7E11 and the four new mAbs can preclear reactivity to one another.

Immunohistochemical Reactivity. The reactivity of mAbs J591, J533, J415, and E99 with normal human tissues and cancers, with rare exception (*vide infra*), were similar to 7E11. Normal tissues with similar immunohistochemical reactivity included prostate (normal and hyperplastic glands demonstrated heterogeneous, weak to moderate staining intensity), kidney (subset of proximal

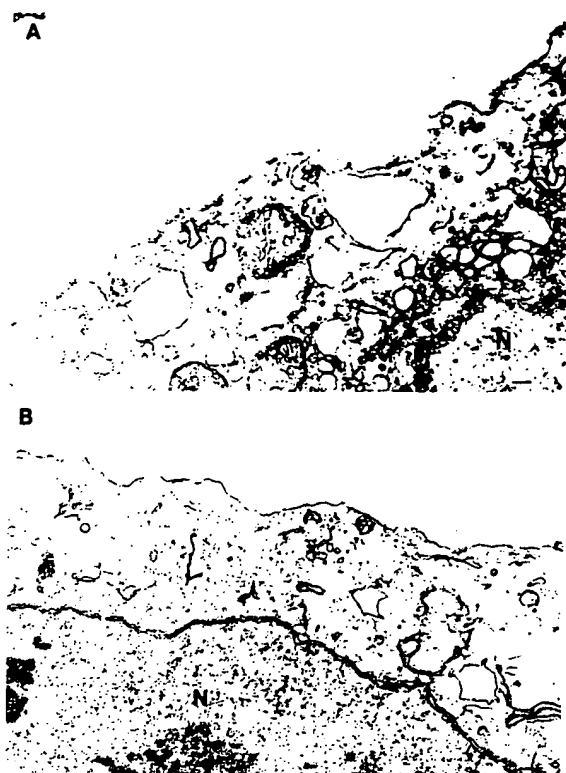


Fig. 4. IEM showing the reactivity of mAbs J591 (A) and 7E11 (B) to viable LNCaP cells. mAb J591 localizes to the extracellular surface of the plasma membrane, whereas 7E11 demonstrates no binding. Bar, 0.6 μ m (A) and 1.0 μ m (B). N, nucleus.

tubules), and duodenum (weakly reactive). The only normal tissue in which we found any difference in reactivity was striated muscle. Although 7E11 was strongly reactive to striated muscle, mAbs J591, J533, J415, and E99 demonstrated no reactivity. In neoplastic tissues, findings were again similar when comparing 7E11 to mAbs J591, J533, J415, and E99. All 21 PCas studied were strongly reactive with mAbs J591, J533, J415, and E99, being somewhat more intense and more homogeneous than 7E11. As reported previously (17), we found 7E11 reacted with vascular endothelium in a subset of tumors. However, mAbs J591, J533, J415, and E99 reacted more strongly with vascular endothelium in all 23 carcinomas studied (Fig. 2), including 9 of 9 renal, 5 of 5 urothelial, 6 of 6 colon, 1 of 1 lung, 1 of 1 breast and 1 of 1 metastatic adenocarcinoma to the liver.

Immunofluorescence Staining of LNCaP Cells. We compared, by indirect immunofluorescence, mAbs J591, J533, J415, and E99 to mAb 7E11 on viable or fixed, permeabilized or nonpermeabilized LNCaP cells (Fig. 3). LNCaP cells with intact plasma membrane (*i.e.*, either viable [data not shown] or fixed without permeabilization) demonstrated cell surface reactivity with mAbs J591, J533, J415, and E99 (Fig. 3, A-C, E, G), but not with mAb 7E11 (Fig. 3f). Only after LNCaP cells were permeabilized could 7E11 reactivity be demonstrated (Fig. 3f). Once permeabilized, the reactivity of all mAbs appeared both in the cytoplasm and on the plasma membrane.

Immunoelectron Microscopy. IEM similarly demonstrated immunoreactivity of mAb J591 (Fig. 4A) but not 7E11 (Fig. 4B) with viable LNCaP cells. Furthermore, the IEM photomicrographs of mAb J591 show the gold particles localized to the extracellular face of the plasma membrane, confirming reactivity with the extracellular domain of PSMA.

Competitive Binding Assay. A double antibody sandwich competition ELISA was used to determine whether the four mAbs recognize the same or different epitopes (Fig. 5). Each unlabeled mAb was able to block its biotinylated counterpart serving as a positive control. An unrelated IgG1 antibody (I56) did not block any of the mAbs to PSMA. J591, J533, and E99 were each able to block each other, but were not blocked by J415. Conversely, J415 was blocked only by its unlabeled counterpart but not by any of the other three mAbs. These results indicate that J591, J533, and E99 recognize the same epitope that is distinct and noncross-reactive with the epitope recognized by J415.

Discussion

This study defines four new IgG mAbs that detect two distinct extracellular epitopes of PSMA (PSMA_{ext1} and PSMA_{ext2}). The reactivity of these mAbs with PSMA has been defined by immunoprecipitation and immunoblotting studies and reactivity against cell lines (data not shown) and tissue sections using the 7E11 mAb as a reference. Immunoprecipitation and immunoblotting studies demonstrate identical reactivity to that seen with 7E11. Reactivity *in vitro* (data not shown) and on tissue sections of normal and neoplastic specimens demonstrates nearly identical results. The exceptions in immunohistochemical reactivity were limited to striated muscle and tumor vascular endothelium. Striated muscle is reactive with 7E11 but not with mAbs J591, J415, J533, or E99. 7E11 reactivity with striated muscle had been reported previously by Lopes *et al.* (4) who, like the present study, used frozen sections but has been reported as negative by Silver *et al.* (17) who studied paraffin sections. This discrepancy is most likely explained by some loss of 7E11/PSMA immunoreactivity in the fixation/embedding process. The difference in reactivity of 7E11 and the present mAbs to striated muscle, both herein studied on frozen sections, may represent differences in the posttranslational processing of PSMA (the external domain of which is heavily glycosylated) occurring in prostate as compared to muscle.

Reactivity of 7E11 with tumor but not normal vascular endothelium also was noted previously by Silver *et al.* (17), although 7E11 reactivity was reported in only half of their renal and urothelial cancers (15 of 30) and 3 of 19 colon carcinomas. In the present study, mAbs J591, J415, J533, and E99 demonstrate reactivity with tumor vasculature in all 23 nonprostate carcinomas tested. Some of the increased reactivity seen herein may represent the benefit of studying frozen as compared to paraffin sections. Within this study, when comparing mAbs J591, J415, J533, and E99 to 7E11 using a constant tissue preparation (frozen sections), we found stronger reactivity with mAbs J591, J415, J533, and E99 than with 7E11. The most likely explanation for the generally stronger reactivity seen with these new mAbs is that they were selected for, among other features, strong immunohistochemical reactivity. We have not studied the immunohistochemical reactivity of mAbs J591, J415, J533, and E99 on paraffin sections.

The initial study with 7E11 (1) indicated reactivity to fixed, but not viable, LNCaP cells later explained by epitope mapping studies indicating the 7E11 epitope to be intracellular (12). A more recent study by Troyer *et al.* (13) studying ultrathin sections by IEM demonstrated 7E11 reactivity on the cytoplasmic aspect of LNCaP

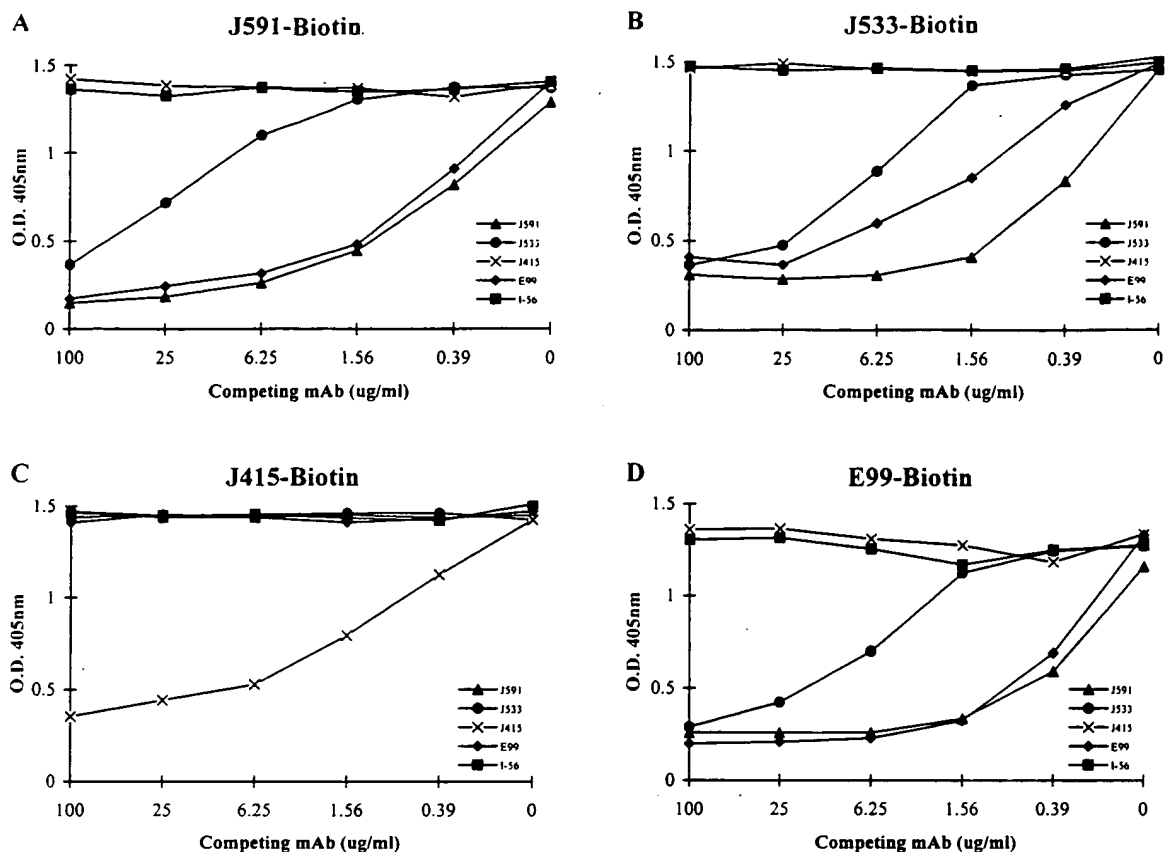


Fig. 5. Competitive binding assay of biotinylated mAbs. PSMA was first captured by 7E11, then unlabeled blocking mAb was added, followed 1 h later by the indicated biotinylated mAb: 0.3 μ g/ml J591-biotin (A); 1.25 μ g/ml J533-biotin (B); 0.2 μ g/ml J415-biotin (C); and 1.25 μ g/ml E99-biotin (D). Each unlabeled mAb blocks its biotinylated counterpart (positive control); mAb I56 (to PSP) did not block (negative control). mAbs J591, J533, and E99 compete with each other for binding, whereas J415 does not.

cells plasma membrane. Troyer *et al.* (13) also confirmed 7E11 reactivity with permeabilized but not with nonpermeabilized LNCaP cells. Our studies comparing 7E11 with the present mAbs by immunofluorescence assays on viable and fixed, permeabilized and nonpermeabilized LNCaP cells confirmed the data published previously that 7E11 detects an intracellular epitope not available for mAb binding unless the cell membrane is disrupted. A recent report by Barren *et al.* (18) represents the sole study indicating that 7E11 can react with viable LNCaP cells. The report by Barren *et al.* is inconsistent with other published work (1, 12, 13), as well as the results reported here, and may be due to a technical point. Barren *et al.*, after incubating 7E11 with viable LNCaP cells, harvested LNCaP for flow cytometry by scraping the cells in the presence of 7E11. As scraping can disrupt cell membranes, this would have provided 7E11 access to its intracellular epitope, which likely accounts for the reactivity reported. Importantly, mAbs J591, J415, J533, and E99, unlike 7E11, can bind to either viable or nonpermeabilized cells consistent with targeting accessible epitopes on the extracellular domain of PSMA. Our IEM finding of mAb J591 localization on the extracellular aspect of the plasma membrane (Fig. 4A), in contrast to the intracellular localization of 7E11 on IEM reported by Troyer *et al.* (13), provides further evidence of reactivity of the present mAbs to the extracellular domain of PSMA.

Epitope mapping of the four present mAbs demonstrates that J591, J533, and E99 each bind to a single epitope (PSMA_{ext1}), whereas J415 binds to a different, noncompeting site (PSMA_{ext2}). This will allow the development of a "sandwich" assay to determine the presence and measure the level of PSMA in serum, which is an area of some current controversy (14, 16).

By allowing the study of viable cells, these mAbs will be useful for studies of PSMA function and PCa cell biology. Recent work indicates that PSMA has glutaminase (19, 20) activity. Studies are under way to determine whether mAbs to PSMA_{ext1} and/or PSMA_{ext2} can block this enzymatic activity and, if so, the effect of such a blockade on normal and neoplastic prostate physiology.

Given prior understanding of PSMA specificity and expression and the established ability of 7E11/CYT-356 to localize *in vivo* to a substantially less available epitope, one would anticipate the likelihood that these new mAbs might demonstrate significantly improved *in vivo* targeting for imaging and therapy. The immunoreactivity of these mAbs to vascular endothelium of a wide variety of cancers may significantly broaden their *in vivo* utility.

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References

- Horoszewicz, J. S., Kawinski, E., and Murphy, G. P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. *Anticancer Res.*, 7: 927-936, 1987.
- Israeli, R. S., Powell, C. T., Fair, W. R., and Heston, W. D. W. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.*, 53: 227-230, 1993.
- Rinker-Schaeffer, C. W., Hawkins, A. L., Su, S. L., Israeli, R. S., Griffin, C. A., Isaacs, J. T., and Heston, W. D. W. Localization and physical mapping of the prostate-specific membrane antigen (PSM) gene to human chromosome 11. *Genomics*, 30: 105-108, 1995.
- Lopes, D., Davis, Wendy, L., Rosenstraus, M. J., Uveges, A. J., and Gilman, S. C. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from antiprostate monoclonal antibody 7E11-C5. *Cancer Res.*, 50: 6423-6429, 1990.
- Israeli, R. S., Powell, C. T., Corr, J. G., Fair, W. R., and Heston, W. D. W. Expression of the prostate-specific membrane antigen. *Cancer Res.*, 54: 1807-1811, 1994.
- Wright, G. L., Jr., Haley, C., Beckett, M. L., and Schellhammer, P. F. Expression of prostate-specific membrane antigen in normal, benign, and malignant prostate tissues. *Urol. Oncol.*, 1: 18-28, 1995.
- Diamond, S. M., Fair, W. R., and Heston, W. D. W. Modulation of prostate specific membrane antigen (PSM) expression *in vitro* by cytokines and growth factors. *Proc. Am. Assoc. Cancer Res.*, 36: 643, 1995.
- Wynant, G. E., Murphy, G. P., Horoszewicz, J. S., Neal, C. E., Collier, B. D., Mitchell, E., Purnell, G., Tyson, I., Heal, A., Abdel-Nabi, H., and Winzelberg, G. Immunoscintigraphy of prostatic cancer: preliminary results with ¹¹¹In-labeled monoclonal antibody 7E11-C5.3 (CYT-356). *Prostate*, 18: 229-241, 1991.
- Murphy, G. P. Radioscintiscanning of prostate cancer. *Cancer* 75 (Suppl.): 1819-1833, 1995.
- Babaian, R. J., Sayer, J., Podoloff, D. A., Steelhammer, L. C., Bhadkamkar, V. A., and Gulfo, J. V. Radioimmunoscintigraphy of pelvic lymph nodes with ¹¹¹indium-labeled monoclonal antibody CYT-356. *J. Urol.*, 152: 1952-1955, 1994.
- Kahn, D., Williams, R. D., Seldin, D. W., Libertino, J. A., Hirschhorn, M., Dreicer, R., Weiner, G. J., Bushnell, D., and Gulfo, J. Radioimmunoscintigraphy with ¹¹¹indium-labeled CYT-356 for the detection of occult prostate cancer recurrence. *J. Urol.*, 152: 1490-1495, 1994.
- Troyer, J. K., Feng, Q., Beckett, M. L., and Wright, G. L., Jr. Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate-specific membrane antigen. *Urol. Oncol.*, 1: 29-37, 1995.
- Troyer, J. K., Beckett, M. L., and Wright, G. L., Jr. Location of prostate-specific membrane antigen in the LNCaP prostate carcinoma cell line. *Prostate*, 30: 232-242, 1997.
- Rochon, Y. P., Horoszewicz, J. S., Boynton, A. L., Holmes, E. H., Barren, R. J., III, Erickson, S. J., Kenny, G. M., and Murphy, G. P. Western Blot assay for prostate-specific membrane antigen in serum of prostate cancer patients. *Prostate*, 25: 219-223, 1994.
- Rajasekaran, A. K., Hojo, M., Huima, T., and Rodriguez-Boulon, E. Catenins and Zonula Occludens-1 form a complex during early stages in the assembly of tight junctions. *J. Cell Biol.*, 132: 451-463, 1996.
- Troyer, J. K., Beckett, M. L., and Wright, G. L., Jr. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int. J. Cancer*, 62: 552-558, 1995.
- Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D. W., and Cordon-Cardo, C. Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res.*, 3: 81-85, 1997.
- Barren, R. J., III, Holmes, E. H., Boynton, A. L., Misrock, S. L., and Murphy, G. P. Monoclonal antibody 7E11.C5 staining of viable LNCaP cells. *Prostate*, 30: 65-68, 1997.
- Pinto, J. T., Suffoletto, B. P., Berzin, T. M., Qiao, C. H., Lin, S., Tong, W. P., May, F., Mukherjee, B., and Heston, W. D. W. Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.*, 2: 1445-1451, 1996.
- Carter, R. E., Feldman, A. R., and Coyle, J. T. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc. Natl. Acad. Sci. USA*, 93: 749-753, 1996.

Biochemical Characterization and Mapping of the 7E11-C5.3 Epitope of the Prostate-Specific Membrane Antigen

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The expression of the prostate-specific membrane antigen (PSMA) glycoprotein recognized by the murine monoclonal antibody (MAb) 7E11-C5.3 has been shown to be highly restricted to prostate epithelium. Although the conjugated form of this MAb (CYT-356) may soon be used clinically for in vivo imaging of extraprostatic disease, few details regarding the nature of the antigenic epitope of PSMA have been reported. This study was carried out to analyze the MAb 7E11-C5.3 epitope on PSMA using standard biochemical techniques, and the antigenic epitope was mapped with synthetic peptides. The MAb 7E11-C5.3 epitope was susceptible to both periodic acid oxidation and proteolytic digestion, which indicated that the antigen consisted of a glycoprotein. However, additional biochemical assays such as sodium borohydride, tunicamycin treatment, and digestion with glycosidases failed to abrogate MAb 7E11-C5.3 binding. Epitope mapping with synthetic peptides demonstrated the epitope to be localized to the intracellular domain at the N-terminus of the PSMA molecule with a minimal reactive peptide consisting of six amino acids (MWNLH). The synthetic peptides were treated with periodic acid, which resulted in inhibition of antibody binding, suggesting that treatment of the PSMA antigen resulted in damage to the peptide chain. These data suggest that the MAb 7E11-C5.3 does not recognize a glycopeptide as was initially thought, but recognizes an intracellular epitope consisting of only the primary polypeptide chain. Further studies are needed to determine how CYT-356 is able to image tumors in vivo when the antigenic epitope is intracellular. (*Urol Oncol* 1995;1: 29-37)

Prostate cancer now exceeds lung cancer as the most commonly diagnosed cancer and is the second leading cause of cancer death in American men.^{1,2} It is estimated that by the year 2000, there will be a 37% increase in prostate cancer deaths and a 90% increase in prostate cancer diagnosis.³ In 1994 alone, there will be an estimated 200,000 new cases reported.⁴ Although an increase in prostate cancer diagnosis may result from improved public awareness and

education as well as screening of at-risk individuals,⁴⁻⁶ a significant challenge will be to maintain the quality of life of those individuals diagnosed with this disease and to identify those patients who are likely to present with recurrent or metastatic disease.

Much has been learned about prostate cancer in recent years, yet there are no effective therapeutic strategies for recurrent prostate carcinoma.⁷ In most cases of recurrent disease, serum prostate-specific antigen (PSA) levels begin to rise before the presence of other evidence of disease as assessed by current diagnostic modalities. As a result, the assumption is made that recurrent disease is present but is not detectable by standard imaging techniques. The clinical use of monoclonal antibodies (MAbs) recognizing prostate-specific biomarkers may provide a significant advance by allowing earlier detection of metastatic foci and site-directed immunotherapeutic approaches in patients with recurrent disease. One such MAb that may prove useful in this regard is the murine MAb 7E11-C5.3, developed using membrane-enriched fractions of the LNCaP prostate carcinoma cell line as an immunogen.⁸ The antigen recognized by MAb 7E11-C5.3 is an approximately 100-kD transmembrane glycoprotein⁹ designated the prostate-specific membrane antigen (PSMA). Recent studies have resulted in the molecular identification and characterization of the gene encoding for this prostate-specific biomarker.¹⁰ The ¹¹¹indium-labeled conjugated form of MAb 7E11-C5.3 (¹¹¹indium CYT-356) has been used to localize LNCaP xenograft tumor growth in nude mice and metastatic prostate carcinoma in humans^{11,12}; it has also been demonstrated to safely detect sites of prostatic carcinoma recurrence after radical prostatectomy.^{13,14} Clinical trials are ongoing to demonstrate further the efficacy of the CYT-356 immunoconjugate for imaging metastatic prostate disease, and studies are underway to determine whether this MAb could be used for targeted therapy approaches.

Preliminary immunohistochemistry studies^{7,15} have indicated that the MAb 7E11-C5.3 epitope may be intracellular, based on the localization of staining and the inability of MAb 7E11-C5.3 to stain living cells. However, CYT-356 is able to image living tumors in vivo, and initial biochemical analysis seemed to indicate that the antigenic epitope contained a significant carbohydrate moiety. These data are conflicting unless the intracellular carbohydrate moiety is protected by an intracellular membrane compartment, because it is unlikely that a carbohydrate would be stable in the cytoplasmic

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environment. At the same time, an intracellular localization of the epitope would make it difficult for CYT-356 to image a living tumor mass in vivo. Because there is some promise that CYT-356 may be a useful tool in clinical practice, it is imperative that these questions regarding the antigenic epitope on PSMA be fully resolved. The purpose of the present study was to determine the physical and biochemical characteristics of the MAb 7E11-C5.3 epitope on this novel prostate-specific biomarker and to precisely map the location of the antigenic determinant. This study confirms that the epitope recognized by the native and immunoconjugated MAb 7E11-C5.3 is intracellular and consists of only the polypeptide chain, and further suggests the sensitivity of antibody-directed imaging; the therapeutic approaches and development of in vitro immunoassays could be enhanced by the production of second-generation antibodies to antigenic epitopes present in the extracellular domain of PSMA.

Materials and Methods

Cells and Reagents

LNCaP cells were obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with L-glutamine, gentamicin (Sigma Chemical Co., St. Louis, MO), and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD). The monoclonal antibody MAb 7E11-C5.3, purified by protein-A affinity chromatography from murine ascites, was provided by Cytogen Corp. (Princeton, NJ). The MAb concentration was determined using a single radial immunodiffusion system (TAGO, Burlingame, CA). Lectins were obtained from E. Y. Laboratories (San Mateo, CA). O-glycanase and N-glycanase enzymes were purchased from Genzyme (Boston, MA). EDAC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide), OPD (0-phenylenediamine dihydrochloride), all other enzymes, carbohydrates, carbohydrate conjugates, and chemicals were purchased from Sigma Chemical Co. unless otherwise noted.

Membrane Preparations

LNCaP cells were harvested and pelleted by centrifugation at $1000 \times g$. The pellet was washed once with ice-cold phosphate-buffered saline (PBS: 136 mmol/L NaCl, 1.7 mmol/L KCl, 8 mmol/L Na_2HPO_4 , 1.5 mmol/L KH_2PO_4 , 0.9 mmol/L CaCl_2 , 0.5 mmol/L MgCl_2 , pH 7.4) and pelleted again. The pellet was resuspended in hypotonic buffer (1 mmol/L NaHCO_3) containing a protease inhibitor cocktail (0.28 mmol/L antipain, 0.75 mmol/L pepstatin, 60 mmol/L ethylenediaminetetra-acetic acid) and incubated on ice for 30 minutes, then dounce homogenized. The homogenate was centrifuged at $2000 \times g$ for 5 minutes in a Beckman JA20 rotor to pellet whole cells and nuclei. The supernatant was collected and centrifuged at $138,000 \times g$ for 2 hours. The supernatant was discarded and the pellet, representing a crude membrane preparation, was resuspended in PBS and stored at -70°C .

PSMA Affinity Purification

Membrane preparations were resuspended in solubilization buffer (30 mmol/L Tris, pH 7.5, 0.5 mol/L NaCl, 1% Nonidet P-40 [NP-40], 0.5% deoxycholate, 0.1% sodium dodecylsulfate

[SDS], 0.5 mmol/L dithiothreitol [DTT]) and protease inhibitor cocktail (as described above) and incubated for 2 hours at 4°C on a rotator. The solubilized material was centrifuged at $100,000 \times g$ for 1 hour at 4°C to pellet nonsolubilized material. The supernatant was collected, diluted 1:2 with 20 mmol/L Tris, pH 8.0, and loaded onto an Affinica Protein A-7E11-C5 affinity column, constructed using the manufacturer's instructions (Schleicher & Schuell, Keene, NH). The column was washed with wash buffer (20 mmol/L Tris, pH 8.0, 0.1% NP-40, 0.1 mmol/L DTT) and eluted with 2N NH_4OH , pH 11.0. The eluted fraction was placed in 3500-MW cutoff dialysis tubing (Spectrum, Houston, TX) and dialyzed against 20 mmol/L Tris HCl, pH 4.0, containing 0.1 mmol/L DTT for 2 hours at 4°C . The eluant was then concentrated against polyethylene glycol compound (MW 15,000-20,000) to approximately 500 μL . Protein concentrations were estimated using the bicinchonic acid (BCA) protein assay, following the manufacturer's instructions (Pierce, Rockford, IL).

Physical and Biochemical Treatment

Purified PSMA from crude LNCaP membrane preparations was boiled for 10 minutes in the presence or absence of mercaptoethanol or SDS. Periodate oxidation¹⁶ and sodium borohydride¹⁷ treatments were carried out as described previously. Proteolytic digestion was achieved by incubating the purified PSMA at 37°C for 24 hours using 100 μL of the following protease solutions: trypsin type III (1, 10, and 100 U/mL), alpha-chymotrypsin type VII (5, 50, and 500 mU/mL), protease type XXI (2, 20, and 200 mU/mL), and protease type XXVI (8, 80, and 800 mU/mL). The treated PSMA was then analyzed using a modified radioimmunoassay (RIA).¹⁸

Glycosidase Treatment

Purified PSMA was treated with beta-galactosidase, fucosidase, endo F, and chondroitinase ABC in Eppendorf tubes, following methods described previously.¹⁹⁻²¹ For the N-glycanase digestion, the antigen was boiled for 3 minutes in the presence of 0.5% SDS and 0.1 mmol/L mercaptoethanol, then diluted in PBS containing 10 mmol/L phenanthroline and NP-40. The N-glycanase (Genzyme, Boston, MA; 0.3 mU) was added and the reaction incubated overnight at 37°C . For the O-glycanase digestion, the antigen was first denatured in SDS and mercaptoethanol as for the N-glycanase treatment, then digested with neuraminidase for 2 hours. O-glycanase (0.5 mU) was added and the reaction mixture incubated overnight at 37°C .

Competitive Binding Experiments

The carbohydrate concentrations were adjusted to 0.05 mmol/L with PBS. The 7E11-C5.3 MAb was incubated with the carbohydrate or PBS control for 2 hours at room temperature. Fifty microliters of the mixture was then used as the primary antibody for an RIA using purified PSMA from LNCaP membrane extract as the antigen. For lectin competitive binding studies, lectins were used at a concentration of 1 mg/mL in PBS. The lectins or PBS control was added to antigen-coated wells in a volume of 100 μL and incubated for 2 hours at room temperature. The wells were washed three times with PBS,

and an RIA was performed as described above. Data for both the carbohydrate and lectin experiments were expressed as a percentage of the control binding using the following formula:

$$\% \text{ control binding} = \frac{\text{CPM of treated well}}{\text{CPM of PBS control well}} \times 100,$$

in which cpm = counts per minute.

Tunicamycin Treatment

LNCaP cells were cultured in the presence of tunicamycin for 7 days, as described previously.²² The cells were harvested and membrane preparations were prepared as described above.

Peptide Synthesis

Peptides were synthesized on a Synergy Peptide Synthesizer (Perkin Elmer-Applied Biosystems, Foster City, CA). Cleavage and extraction of the crude peptide were performed according to the manufacturer's instructions. All peptides gave a single discrete peak when analyzed by reverse-phase chromatography on a Waters 650 high-performance liquid chromatography system. The sequence of the peptides was derived from the published nucleotide sequence of PSMA¹⁰ and named according to the distance from the N-terminus of the deduced amino acid sequence (Table 1).

Direct Binding Peptide Assay

Peptides were bound to wells of microtiter plates using the procedure described previously,²³ with several modifications. Briefly, 100 μ L of bovine serum albumin (BSA) (1 μ g/mL) was dispensed into each well of a high-binding enzyme immuno-

assay (EIA) plate (Costar, Cambridge, MA) and dried overnight. The plates were rinsed twice with PBS, followed by two rinses with distilled water. Various concentrations of peptides were added to the wells in a volume of 50 μ L, followed by 50 μ L of EDAC cross-linker (10 mg/mL) or water control, and the plates were incubated overnight at 4°C with shaking. The plates were washed five times with wash buffer (PBS containing 0.05% Tween-20). The concentration of peptides adhered to the plate was determined by protein assay of the peptide-EDAC mixture before and after the cross-linking incubations. A standard enzyme-linked immunosorbent assay (ELISA) was then performed (as described below).

Competitive Binding Peptide Assay

Competition plates were made by first dispensing 100 μ L BSA (1 μ g/mL) into the wells of a high-binding EIA plate and drying overnight. The plate was rinsed twice with PBS and twice with distilled water before the addition of peptides. Serial 1:5 dilutions of each peptide were made with water, starting at a concentration of 1 mmol/L and with a final volume of 40 μ L in each well. The antibody concentration used was determined by finding the concentration of Mab 7E11-C5.3 that gave half maximal binding to a constant concentration of purified PSMA antigen at 200 ng per well, determined to be 1 μ g/mL. Forty microliters of Mab 7E11-C5.3 (1 μ g/mL) was added to each well, and the plate was incubated at 4°C overnight with shaking.

Antigen plates were prepared by dispensing 100 μ L of purified PSMA (2.0 μ g/mL) into the wells of high-binding EIA plate (Costar) and incubating overnight at 4°C with shaking. The antigen plates were washed four times with wash buffer and blocked with blocking buffer (1% BSA in PBS containing

TABLE 1. AMINO ACID SEQUENCES OF SYNTHETIC PSMA PEPTIDES

Name*	Amino acid sequence
N1.19	M W N L L H E T D S A V A T A R R P R
N1.12	M W N L L H E T D S A V
N1.11	M W N L L H E T D S A
N1.10	M W N L L H E T D S
N1.9	M W N L L H E T D
N1.8	M W N L L H E T
N1.7	M W N L L H E
N1.6	M W N L L H
N1.5	M W N L L
N1.4	M W N L
N2.6	W N L L H
N7.12	E T D S A V
N13.19	A T A R R P R
NΔ1.6	C W N L L H
N1.6Δ	M W N L L Y
N344	M H I H S T
N470	M Y S L V H
N583	M V F E L A
N664	M N D Q L M
N669	M F L E R A

*Named according to the distance from the N-terminus of the PSMA amino acid sequence.

0.05% Tween-20) for 1 hour at room temperature. The blocking buffer was removed, and the 80 μ L of the peptide-antibody mixture from the inhibition plates prepared above was transferred to the antigen plates and used as the primary antibody for a standard ELISA.

Standard ELISA

Microtiter plates, activated with peptide or antigen, were blocked for 1 hour at room temperature with blocking buffer (1% BSA in PBS containing 0.05% Tween-20) with shaking. The blocking buffer was removed, and Mab 7E11-C5.3 or competition mix (as described above) was added to each well and incubated for 2 hours at room temperature with shaking. The plates were washed five times with wash buffer, with the final wash being removed by vacuum aspiration to ensure that all of the wash buffer was removed. The secondary antibody (1 μ g/mL horseradish peroxidase-labeled, horse anti-mouse antibody; Vector Laboratories, Burlingame, CA) was added at 50 μ L per well and incubated for 2 hours at room temperature with shaking. The plates were washed five times with wash buffer, and all of the buffer was removed with the final wash. After the final wash, 200 μ L of OPD substrate was added to each well and incubated for 5-30 minutes with shaking, and read at 405 nm on an EL-340 Microplate reader (Bio-Tek Instruments, Winooski, VT).

Determination of Affinity Constant (K_d)

The affinity of Mab 7E11-C5.3 for the native antigen and peptides was determined by a modification of a method described previously.²⁴ The MAb binding was plotted as bound/free antibody as a function of the concentration of bound antibody (mmol/L). The regression coefficient (K_d) was calculated according to the method of Scatchard,²⁵ and the Student *t* test was applied to determine the significance of the results.

Results

Basic Biochemical Characterization of the PSMA Epitope

The initial characterization of the PSMA epitope used standard physical and biochemical techniques on purified material. The antigen was stable after incubations at 100°C and after reduction or denaturation in mercaptoethanol and SDS (Figure 1A). The epitope was susceptible to oxidation of vicinyl hydroxyl groups by periodic acid, suggesting a carbohydrate component of the epitope, although sodium borohydride (Figure 1A) and tunicamycin treatments (data not shown) were unable to inhibit binding. The increased binding of Mab 7E11-C5.3 to PSMA after sodium borohydride treatment most likely results from the release of O-linked carbohydrates, which may mask the antigenic determinant.

The recognition of the PSMA epitope by Mab 7E11-C5.3 under reducing and denaturing conditions indicates that the integrity of the epitope was not dependent on the secondary or tertiary structure of the native molecule. Treatment of PSMA with a variety of proteases having differing specificities revealed the epitope to be highly susceptible to proteolytic digestion (Figure 1B).

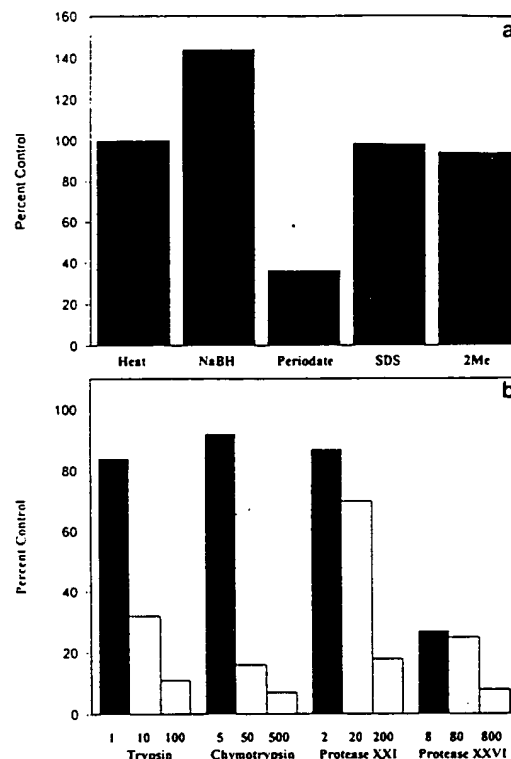


FIGURE 1. Basic physical and biochemical analysis of the PSMA epitope. A) Treatment of purified PSMA from LNCaP membrane extracts with heat (100°C for 10 minutes), sodium borohydride (NaBH), periodate, sodium dodecyl sulfate (SDS), and mercaptoethanol (2Me). The antigen was recognized in a denatured and reduced form and was susceptible to periodate oxidation but not sodium borohydride treatment. B) PSMA from LNCaP membrane extracts was highly susceptible to proteolytic digestion with trypsin (U/mL), chymotrypsin (mU/mL), protease type XXI (mU/mL), and protease type XXVI (mU/mL).

Carbohydrate Analysis

To determine whether the antigenic epitope contained a carbohydrate moiety, we performed lectin inhibition studies. These experiments demonstrated that SBA, PNA, and MPA lectins, which are specific for D-galactose in either a monomeric form or a polymeric form, were able to reduce Mab 7E11-C5.3 binding, whereas lectins that bind to other carbohydrates such as mannose, glucose, fucose, neuraminic acid, and N-acetyl-glucosamine had no effect (Figure 2A). To prove the specificity of the lectin experiments, we used both general and specific glycosidases to digest PSMA. Neither O-glycosidase nor N-glycosidase, used to cleave O-linked and N-linked oligosaccharides, respectively, were able to reduce Mab 7E11-C5.3 binding. Digestion with specific enzymes, in particular beta-galactosidase, also did not inhibit antibody binding (Figure 2B). Similarly, Mab 7E11-C5.3 binding was not inhibited in competitive blocking experiments using specific car-

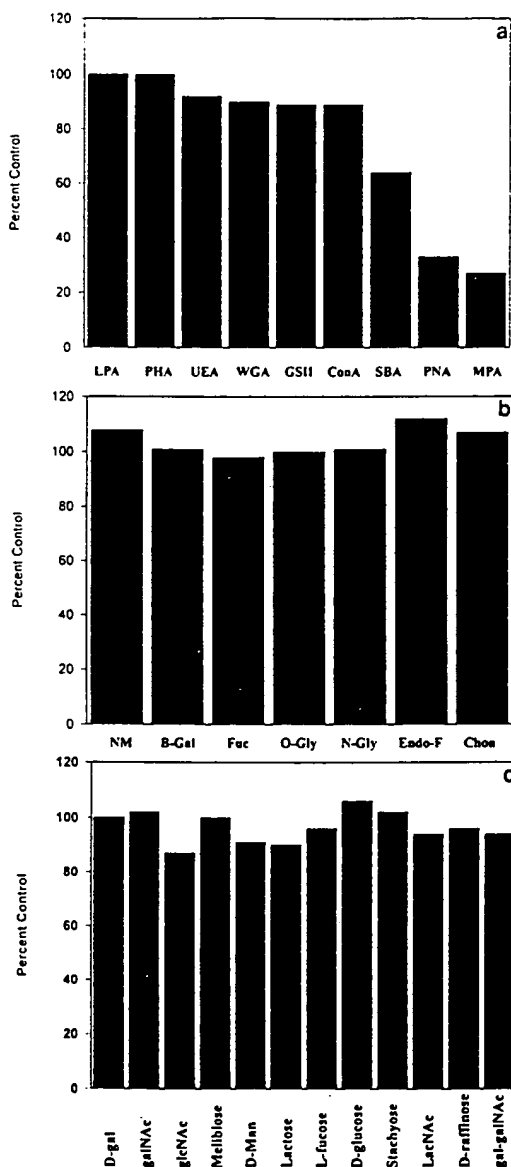


FIGURE 2. Carbohydrate analysis of the PSMA epitope. A) Competitive lectin-binding experiments showing inhibition with SBA, PNA, and MPA lectins. The carbohydrate specificities for the lectins are as follows: LPA (NeuNAc, D-galNAc, and D-GlcNAc), PHA (oligosaccharides), UEA (α -L-fuc), WGA (D-glcNAc, NeuNAc), GSII (D-glcNAc), ConA (α -D-man, α -D-glc), SBA (α -D-galNAc, D-gal), PNA (D-gal- β -(1-3)-D-galNAc), and MPA (α -D-gal). B) Digestion of PSMA from LNCaP membrane extracts with neuraminidase (NM), β -galactosidase (B-Gal), fucosidase (Fuc), O-glycanase (O-Gly), N-glycanase (N-Gly), Endo F, and chondroitinase ABC (Chon). C) Competitive blocking with specific carbohydrates and aminosugars, demonstrating the inability to block MAb 7E11-C5.3.

bohydrates or aminosugars (Figure 2C). In particular, D-galactose (D-gal) and N-acetyl galactosamine (galNAc) were unable to inhibit binding. These saccharides correspond to the specificities of the SBA, PNA, and MPA lectins, which were able to reduce binding. The inability of these sugars and glycosidases to abrogate binding indicates that the lectin inhibition may be nonspecific.

Peptide Binding

Because anecdotal evidence suggested that the PSMA epitope was intracellular, several synthetic peptides were synthesized to correspond to the 19 amino acids of the proposed intracellular domain of PSMA. Peptide N1.19 consisted of the entire 19-amino acid sequence (MWNLHETDSAVATARRPR), whereas peptides N1.6 (MWNLH), N7.12 (ETDSAV), and N13.19 (ATARRPR) were synthesized to correspond to three segments of N1.19. Figure 3 shows that N1.19 and the N-terminal N1.6 peptides were recognized by MAb 7E11-C5.3, although N1.6 had approximately 30% less binding than N1.19. N7.12, N13.19, and N669 (MFLERA), a negative control peptide, were inactive, indicating that the PSMA epitope was predominantly composed of the core peptide structure and located at the amino terminal end of the glycoprotein.

Epitope Mapping

To characterize further the peptide portion of the PSMA epitope, we mapped the intracellular epitope completely. Ta-

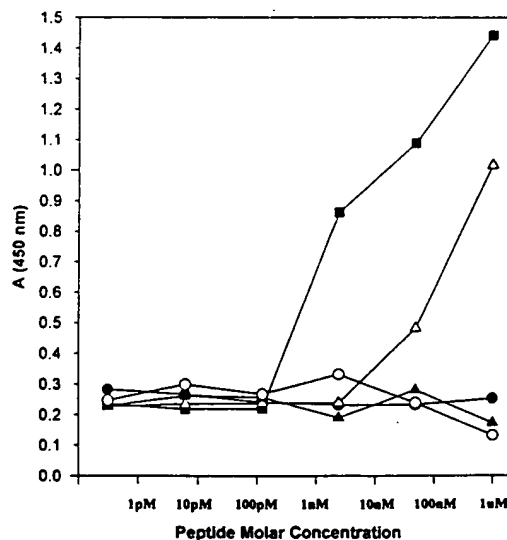
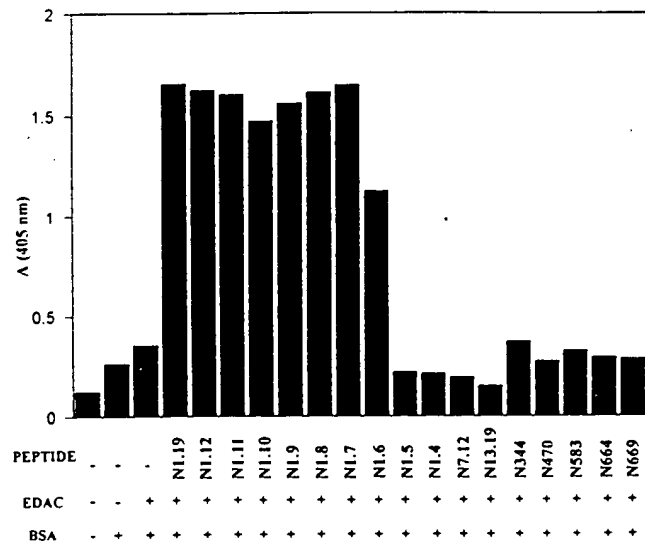


FIGURE 3. Direct-binding PSMA peptide assay with 50 μ L of each molar concentration of peptide. Peptides were bound to ELISA plates with BSA and EDAC chemical cross-linker and used for a standard ELISA with MAb 7E11-C5.3. The concentration of MAb 7E11-C5.3 used (1 μ g/mL) was determined analytically from the half-maximal binding to the purified antigen. Peptides N1.19 (solid squares) and N1.6 (open triangles) were active in a dose-dependent manner, whereas peptides N7.12 (closed circles), N13.19 (closed triangles), and the control peptide N669 (open circles) were negative.

FIGURE 4. PSMA peptide mapping using 50 μ L of each peptide at a concentration of 1 μ mol/L. Baseline levels were defined by control wells containing only bovine serum albumin (BSA) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), or both. All peptides containing at least the first seven amino acids were equally active, with N1.6 activity dropping by approximately one third. The methionine-containing negative control peptide N669 was below baseline, as were additional peptides from the PSMA amino acid sequence, which had a motif similar to N1.6.



ble 1 shows the orientation of the synthetic peptides, starting with the complete N1.19 peptide. Equimolar amounts of each peptide were assayed in a direct binding assay. All of the peptides containing the first seven amino acids had equal activity (Figure 4), whereas N1.6 retained approximately two thirds the activity and N1.5 was below baseline levels. These results indicate that the minimal reactive peptide was a 6-mer composed of the first six amino terminal amino acids (MWNLH). Several additional peptides within the proposed PSMA amino acid sequence had a motif similar to N1.6, including N344, N470, N583, and N664. These peptides were also synthesized and analyzed, but were not bound by Mab 7E11-C5.3 (Figure 4). To demonstrate the specificity of the synthetic peptides and to use an alternative method that did not require binding of the peptides to the microtiter plates, we performed an indirect assay to block antibody binding competitively. Peptides N1.19 and N1.6 were able to inhibit binding of Mab 7E11-C5.3 to PSMA in a dose-dependent manner, whereas control peptides and other peptides present in the PSMA sequence were unable to compete for binding (Figure 5).

Biochemical Analysis of the Antigenic Peptides

To determine whether the decrease in antibody binding after periodate treatment of the purified PSMA in Figure 1 resulted from the oxidation of the peptide epitope and not a carbohydrate, the synthetic peptides were treated with periodate before binding to the EIA plate for the direct binding assay. Periodate treatment of peptides N1.19 and N1.6 abrogated Mab 7E11-C5.3 binding (Figure 6). In addition, to mimic the effects of periodate oxidation of the polypeptide chain, peptide N2.6 (WNLLH), with a deletion of the methionine residue, or NΔ1.6 (CWNLH), with a substitution of cysteine for the

methionine residue, as well as peptide N1.5 (MWNL), with a deletion of the histidine residue, or N1.6Δ (MWNL), with a substitution of tyrosine for the histidine residue, were synthesized and assayed in a direct binding assay. The deletion

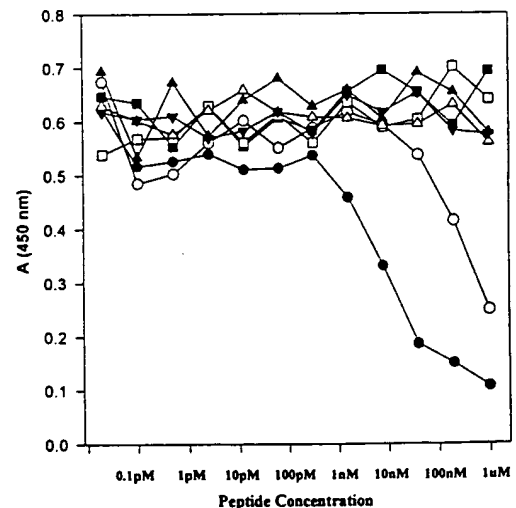


FIGURE 5. PSMA peptide inhibition assay. Forty microliters of each peptide dilution was incubated with 40 μ L of the Mab 7E11-C5.3 and then used as the primary antiserum for an ELISA with purified PSMA from LNCaP cells as the antigen. Peptides N1.19 (closed circles) and N1.6 (open circles) competed in a dose-dependent manner, whereas the negative control peptide N669 (open squares) and peptides N334 (upward triangles), N470 (downward triangles), N583 (closed squares), and N664 (open triangles) were unable to block Mab 7E11-C5.3.

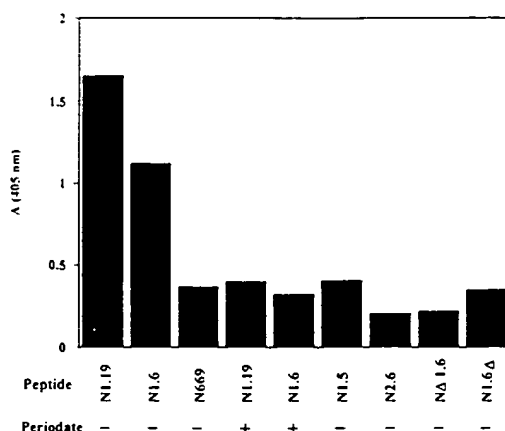


FIGURE 6. Analysis of the MAb 7E11-C5.3 epitope. Peptides N1.19 and N1.6 were treated with periodate as described for the native PSMA antigen. The activity of both peptides was reduced to the background levels of the negative control peptide (N669). In addition, deletion of the methionine (N2.6) and histidine (N1.5) or substitution of the methionine with cysteine (NΔ1.6) and substitution of the histidine with tyrosine (N1.6Δ) resulted in a similar loss of activity.

of either of these amino acids resulted in a loss of activity (Figure 6), suggesting that they or at least the amino acids present in that position are important for the structure of the epitope. Substitution of the methionine with cysteine and of the histidine with tyrosine also resulted in a loss of activity, indicating that the amino-terminal methionine and the carboxy-terminal histidine are required for structural integrity of the PSMA epitope and are essential for MAb 7E11-C5.3 activity, which, if lost by oxidation, would result in an abrogation of MAb 7E11-C5.3 binding.

The affinity of MAb 7E11-C5.3 was determined for the native PSMA glycoprotein and for the N1.19 and N1.6 active peptides. The affinity for the native PSMA glycoprotein (K_d $1.16 \times 10^{10} \text{ M}^{-1}$) was approximately 6.8-fold higher than the affinity for the N1.19 peptide (K_d $1.7 \times 10^9 \text{ M}^{-1}$) and about 50-fold higher than that for the N1.6 peptide (K_d $2.3 \times 10^8 \text{ M}^{-1}$), although the affinity for N1.6 was only 7.4-fold lower than the affinity for N1.19.

Discussion

The prostate-specific membrane antigen (PSMA) consists primarily of a major Mr 120,000 protein in tissue extracts and seminal plasma and an Mr 100,000 protein in LNCaP membrane extracts by Western blot analysis.^{9,26,27} The amino acid sequence for the Mr 100,000 species expressed in the LNCaP cell line indicates that PSMA is a transmembrane glycoprotein.^{10,28} Immunohistochemistry studies have demonstrated that PSMA is expressed in normal, benign, and malignant prostate epithelial cells but not in other normal adult tissues, with the possible exception of skeletal muscle,^{8,11} which appears to show nonspecific staining (unpublished data). Over-

all, the data reported thus far continue to suggest that PSMA is a novel antigen with expression highly restricted to prostate tissues and that it may prove to be a useful biomarker for targeted diagnostic or therapeutic strategies or for gene therapy approaches. The MAb 7E11-C5.3 is the only reported antibody reactive to this glycoprotein, and it is therefore of interest to determine the epitope on PSMA that is reactive to this MAb. It is especially important to clarify the questions regarding the nature of the antigenic epitope with respect to the biochemical nature and location, as they could directly influence our understanding of the ability of CYT-356 to be useful in radioimmunoscintigraphy and immunotherapy. The present study examined the biochemical nature of PSMA and the epitope recognized by MAb 7E11-C5.3.

The basic physical and biochemical analysis of the PSMA epitope revealed that it is recognized in both a reduced and a denatured state based on the activity of the antigen in mercaptoethanol and SDS, respectively. Treatment of antigens with periodic acid has been used in the past to indicate the presence of carbohydrates in antigenic epitopes.¹⁶ Therefore, the loss of activity after periodate treatment along with the sensitivity of PSMA to proteolytic digestion was initially interpreted to mean that the epitope consisted of a glycopeptide. In an attempt to determine the type of carbohydrate linkage in the epitope, the antigen was treated with sodium borohydride to cleave O-linked oligosaccharides; alternatively, LNCaP cells were grown in the presence of tunicamycin before antigen preparation to inhibit the addition of N-linked polysaccharides to the nascent peptide chain in the endoplasmic reticulum. Neither of these more specific treatments had any effect on MAb 7E11-C5.3 activity, contradicting the periodate results.

Lectin-binding experiments also indicated that carbohydrates were present in the epitope and that this carbohydrate component contained D-galactose in some form, because only lectins that were specific for either monomeric or polymeric galactose were able to block MAb 7E11-C5.3 binding. However, as with the basic biochemical analysis, we were unable to demonstrate specificity by either glycosidic digestion of PSMA or competitive binding experiments using specific monomeric or polymeric carbohydrates or aminosugars.

Because there was evidence to suggest that the antigenic epitope was intracellular, and considering the fact that there were only 19 amino acids in the intracellular domain,¹⁰ peptides were synthesized to span this domain. The full-length 19-mer (N1-19) was active in both direct and competitive binding assays in a dose-dependent manner. Mapping of the peptide epitope by sequentially deleting residues from N1.19, one amino acid at a time, demonstrated that the minimal reactive peptide consisted of the first six N-terminal amino acids (MWNLLH). Deletion and substitution of the amino-terminal methionine or the carboxy-terminal histidine residue abrogated MAb 7E11-C5.3 binding, proving that these amino acids are essential for the integrity of the epitope. The affinity of MAb 7E11-C5.3 for N1.19 (K_d $1.7 \times 10^9 \text{ M}^{-1}$) was approximately 6.8-fold lower than the affinity for the native PSMA glycoprotein (K_d $1.16 \times 10^{10} \text{ M}^{-1}$), whereas the affinity of MAb 7E11-C5.3 for N1.6 (K_d $2.3 \times 10^8 \text{ M}^{-1}$) was 7.4-fold lower than the affinity for N1.19. The direct peptide assays used in this study are a convenient means of assaying MAb

binding to peptides, which would otherwise be unable to adhere to the plastic of the assay plates, by covalently cross-linking the peptides with EDAC to BSA in the wells of the EIA plate. However, the peptides are covalently linked to BSA, which may significantly alter their conformation as compared to the native PSMA. It is not surprising or unprecedented, then, that the affinity constants for the peptides and the native antigen are slightly different.²⁹ This difference may be explained not only by the effects of cross-linking of the peptides to BSA, as discussed above, but also in differences between the linear synthetic peptides and the native PSMA molecule, which may have a significantly different conformation. In addition, modifications of the amino acids in the native protein, such as acetylation or other additions, might further stabilize the antigenic determinant of the native PSMA. Although the affinity of the N1.6 peptide may be lower than that of N1.19 and the native PSMA molecule for the same reasons as discussed above, the inhibition studies (Figure 5) seem to suggest that the smaller peptide does indeed have a lower affinity which most likely results from a destabilization of the antigenic determinant. Nevertheless, the epitope-mapping studies clearly demonstrate that the epitope recognized by MAb 7E11-C5.3 is the primary peptide chain of the intracellular domain of PSMA and that the minimal reactive peptide consists of the first six amino acids (MWNLH) at the amino terminus.

It is important to note that several peptides are present in the extracellular domain of PSMA, which contains a motif similar to that of N1.6 (methionine at position 1 and histidine or a similar amino acid at position 6), but none of these peptides were active by direct binding or competitive binding assays. There was a possibility that these small peptides were not bound to the microtiter plates as efficiently as larger peptides. However, the competitive binding assay did not require that the peptides be bound and was an important control experiment to verify the negative results for these peptides observed in the direct binding assay. The size of the peptides may, however, cause a decrease in MAb 7E11-C5.3 affinity, as seen between N1.19 and N1.6, and experiments are underway to determine whether larger peptides from these regions are active.

The suggestion from the epitope-mapping data that the epitope consisted of only the core peptide and did not contain a carbohydrate moiety would explain the failure of the sodium borohydride, tunicamycin, and glycosidase treatments and of specific carbohydrates to block the MAb 7E11-C5.3 activity. However, the periodate oxidation and lectin competitive binding experiments were still contradicting if the MAb 7E11-C5.3 only binds to the peptide chain. Although periodate oxidation has been used successfully to identify carbohydrate epitopes, oxidation of amino acids also may occur.³⁰ The treatments used in this study (10 mmol/L periodate for 1 hour at 25°C) should have been mild enough to preclude damage to the polypeptide chain, while nonreducing sugars and pyranosidically linked hexoses within the oligosaccharide chains would have been oxidized.¹⁶ Harsher conditions including longer incubations, higher temperatures, or higher periodate concentrations could have resulted in destruction of the peptide chain in addition to any carbohydrate. The possibility existed that the PSMA peptide

epitope was particularly sensitive to periodate. This was especially possible because methionine residues are particularly susceptible to periodate oxidation.³⁰ In addition, the reactive peptides all began with the very N-terminal methionine of the protein, which may make it even more subject to oxidative attack because there is no protection on the amino-terminal end of this amino acid. Treatment of the reactive peptides N1.19 and N1.6 with periodate did, in fact, abrogate MAb 7E11-C5.3 binding. Oxidation of the peptide may result in cleavage or alteration of the amino acid residues. Therefore, to demonstrate the effects of either a loss or change in structure of the amino acids, peptides were synthesized with deletions or substitutions of either the methionine or the histidine residues, which also resulted in a loss of antibody binding. Clearly, both the methionine and the histidine residues were essential for activity of the peptides, and these experiments suggest that periodate oxidation was affecting the primary amino acid structure of PSMA.

Although it has been reported that PSMA can be detected in serum,^{8,31} we and others have been unable to duplicate these results (unpublished data). The sensitivity of both the peptides and the native PSMA molecule to oxidation may explain the inability of MAb 7E11-C5.3 to detect the antigen in serum in our hands, as a result of either cleavage or blockage of the amino terminus by any number of serum factors. Experiments are underway to determine whether the native antigen, purified from LNCaP cells, or active PSMA peptides are stable in serum for extended periods. These data suggest that serum assays would be greatly improved if second-generation MAb were created against more stable epitopes.

The ability of some lectins to block the MAb 7E11-C5.3 binding may have occurred as a result of nonspecific binding, because the native PSMA does contain a substantial amount of carbohydrate. The deglycosylated polypeptide has an Mr of 84,000, and the fully glycosylated molecule has an Mr of 100,000.²⁸ In addition, only lectins with a specificity for D-galactose were able to block MAb 7E11-C5.3 binding, although D-galactose in either monomeric or polymeric form was unable to compete for the MAb. The latter finding suggests that there may be a significant amount of galactose present on the native PSMA glycoprotein, but not in the antigenic epitope. If a number of D-galactose-containing oligosaccharides were close to but not directly in the antigenic epitope, the lectins could nonspecifically block MAb 7E11-C5.3 by steric hindrance and not by direct competition.

Although we have not mapped the entire PSMA molecule, the results of this study convincingly show that the epitope recognized by MAb 7E11-C5.3 is located in the intracellular domain of the PSMA transmembrane glycoprotein and consists solely of the polypeptide chain with a minimal reactive unit consisting of six amino acid residues (MWNLH). An intracellular location of the MAb 7E11-C5.3 epitope introduces questions regarding the ability of CYT-356 to image living tumor masses. The immunoconjugated form of MAb 7E11-C5.3 (CYT-356) has been shown clearly to detect solid tumors *in vivo*¹¹⁻¹⁴ even though the epitope is intracellular. Possible explanations for this observation may be that CYT-356 images only necrotic tissues or those cells that have been lysed, either mechanically or via apoptosis, or that CYT-356 may be able to cross the plasma membrane to bind the an-

tigen. Alternatively, several peptides are present within the extracellular domain of PSMA: N470 (MYSLVH), N344 (MHIHST), N583 (MVFELA), and N664 (MNDQLM), which contain a motif similar to that of the N1.6 minimal reactive peptide. Although these peptides were not active by either a direct binding assay or a competitive binding assay, it is possible that CYT-356 may have a low affinity for any one of these peptides, which might result in a detectable signal upon immunoscintigraphy. Clearly, more studies are needed to determine definitively how CYT-356 is able to image solid tumors. Based on our findings that Mab 7E11-C5.3 binds to a peptide epitope localized to the intracellular domain, it would appear highly advantageous to produce second-generation antibodies to antigenic epitopes present in the extracellular domain of this transmembrane glycoprotein. Such antibodies could conceivably enhance the sensitivity of antibody-directed imaging and therapy applications, as well as provide excellent reagents for the development of in vitro diagnostic tests.

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References

- Boring CC, Squires TS, Tong T, Montgomery S. Cancer statistics. *CA* 1994;44:7-26.
- Colley DS. Prostate cancer: An overview of an increasing dilemma. *Cancer* 1993;71(suppl):880-6.
- Carter HB, Coffey DS. The prostate: An increasing medical problem. *Prostate* 1990;16:187-97.
- Lange PH, Winfield HN. Biological markers in urologic cancer. *Cancer* 1987;60:464-72.
- Cupp MR, Oesterling JE. Prostate specific antigen, digital rectal exam, and transrectal ultrasonography: Their roles in diagnosing early prostate cancer. *Mayo Clin Proc* 1993;68:297-306.
- Stormont TJ, Farrow GM, Myers RP, et al. Clinical stage B0 or T1c prostate cancer: Nonpalpable disease identified by elevated serum prostate-specific antigen concentration. *Urology* 1993;41:3-8.
- Schellhammer PF, Kuban DA, el-Mahdi AM. Local failure after definitive radiation or surgical therapy for carcinoma of the prostate and options for prevention and therapy. *Urol Clin North Am* 1991;18:495.
- Horoszewicz JS, Kawinski E, Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostate cells and serum of prostatic cancer patients. *Anticancer Res* 1987;7:927-36.
- Wright GL Jr, Feng Q, Lipford GB, Lopes D, Gilman SC. Characterization of a new prostate carcinoma-associated marker: 7E11-C5. Fifth International Conference on Monoclonal Antibody Immunocytogenetics for Cancer 1990;3:89 (abstract 193).
- Israeli RS, Powell CT, Fair WR, Heston DW. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res* 1993;53:227-30.
- Lopes AD, Davis WL, Rosenstraus MJ, Uveges AJ, Gilman SC. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from anti-prostate monoclonal antibody 7E11-C5. *Cancer Res* 1990;50:6423-9.
- Wynant GE, Murphy GP, Horoszewicz JS, et al. Immunoscintigraphy of prostatic cancer: Preliminary results with ¹¹¹In-labeled monoclonal antibody 7E11-C5.3 (CYT-356). *Prostate* 1991;18:229-41.
- Kahn D, Williams RD, Seldin DW, et al. Radioimmunoscintigraphy with ¹¹¹indium labeled CYT-356 for the detection of occult prostate cancer recurrence. *J Urol* 1994;152:1490-5.
- Babaian RJ, Sayer J, Podoloff DA, Steelhammer LC, Bhadkamkar VA, Gullo JV. Radioimmunoscintigraphy of pelvic lymph nodes with ¹¹¹indium-labeled monoclonal antibody CYT-356. *J Urol* 1994;152:1952-5 (abstract).
- Troyer JK, Adam M, Wright GL Jr. Subcellular localization of the 7E11-C5 prostate specific antigen. *Proc Am Assoc Cancer Res* 1994;35:283 (abs. 1688).
- Woodward MP, Young WW, Bloodgood RA. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods* 1985;78:143-53.
- Sekine H, Ohno T, Kule DW. Purification and characterization of a high molecular weight glycoprotein detectable in human milk and breast carcinomas. *J Immunol* 1985;135:3610-5.
- Starling JJ, Sieg SM, Beckett ML, et al. Human prostate tissue antigens defined by murine monoclonal antibodies. *Cancer Res* 1986;46:367-74.
- Li SC, Mazzotta MY, Chien SF, Li YT. Isolation and characterization of Jack Bean Beta-galactosidase. *J Biol Chem* 1975;250:6786-91.
- Tarentino AL, Gimez CM, Plummer TH Jr. Deglycosylation of asparagine linked glycans by peptide N-glycosidase F. *Biochemistry* 1985;24:4665-71.
- Tatsuya Y, Saito H, Habuchi O, Suzuki S. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J Biol Chem* 1968;243:1523-35.
- Banerjee R, Ginsburg E, Vonderhaar BK. Characterization of a monoclonal antibody against human prolactin receptors. *Int J Cancer* 1993;55:712-21.
- Price MR, Sekowski M, Hooi DSW, Durrant LG, Hudecz F, Tendler SJB. Measurement of antibody binding to antigenic peptides conjugated in situ to albumin-coated microtiter plates. *J Immunol Methods* 1993;159:277-81.
- Howlett BJ, Clarke AE. Detection of lectin binding to glycoconjugates immobilized on polyvinyl chloride microtitre plates. *Biochem Int* 1981;2:553-60.
- Scatchard G. The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* 1949;51:660-72.
- Feng Q, Beckett ML, Kaladas P, Gilman S, Wright GL Jr. Purification and biochemical characterization of the 7E11-C5 prostate carcinoma-associated antigen. *Proc Am Assoc Cancer Res* 1991;32:239 (abstract 1418).
- Troyer JK, Feng Q, Beckett ML, Morningstar MM, Wright GL Jr. Molecular characterization of the 7E11-C5 prostate tumor-associated antigen. *J Urol* 1993;149:333A (abs. 482).
- Israeli RS, Powell T, Corr JG, Fair WR, Heston DW. Expression of the prostate-specific membrane antigen. *Cancer Res* 1994;54:1807-11.
- Xing PX, Reynolds K, Tjandra JJ, Tang XL, McKenzie IFC. Synthetic peptides reactive with anti-human milk fat globule membrane monoclonal antibodies. *Cancer Res* 1990;50:89-96.
- Clamp JR, Hough L. The periodate oxidation of amino acids with reference to studies on glycoproteins. *Biochem J* 1965;94:17-24.
- Rochon YP, Horoszewicz JS, Boynton AL, et al. Western blot assay for prostate-specific membrane antigen in serum of prostate cancer patients. *Prostate* 1994;25:219-23.

DETECTION AND CHARACTERIZATION OF THE PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA) IN TISSUE EXTRACTS AND BODY FLUIDS

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The prostate-specific membrane antigen (PSMA) glycoprotein is recognized by the monoclonal antibody (MAb) 7E11-C5.3 as a predominant 100 kDa and minor 180 kDa component in LNCaP cell line extracts and its expression has been shown by immunohistochemistry to be highly restricted to prostate epithelium. The aim of the present study was to utilize Western blot analysis to determine if PSMA could be detected in human tissue extracts and body fluids and if so, which molecular forms were present. PSMA was detected as 120 and 200 kDa bands in normal, benign and malignant prostate tissues and seminal plasma. Further analysis demonstrated that the larger molecular form of PSMA may be a dimer of the lower m.w. species. The PSMA glycoprotein was not detected in the majority of non-prostate tissue extracts examined except for a low yet significant amount in normal salivary gland, brain and small intestine, suggesting that PSMA may not be as prostate-specific as originally thought. Since the prostate-specific antigen (PSA) has been shown to be maximally shed into the serum in high-grade and metastatic prostate carcinomas, it was surprising that PSMA could not be detected in serum by Western blot analysis even in patients with actively progressive metastatic disease. Second generation antibodies generated against different epitopes may be required to determine if PSMA is shed into serum. Our results support the hypothesis that PSMA is a novel prostate biomarker.

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Prostate cancer (CaP) is the most common malignancy and the second leading cause of cancer death in males, with an estimated 244,000 new cases and 40,400 deaths in 1995 (Wingo *et al.*, 1995). As with any malignancy, early detection and treatment are paramount in reducing mortality in prostate carcinoma patients. The combined use of serum prostate-specific antigen (PSA) measurements and digital rectal examination have markedly enhanced the detection rate of prostate cancer. However, approximately 30% of benign prostatic hyperplasia (BPH) patients give false-positive PSA levels, and 25-30% of CaP patients have normal serum PSA concentrations (Cupp and Oesterling, 1993). These findings, and the recent controversy regarding whether and when to treat and what treatment modality to use, suggest that additional biomarkers need to be identified to differentiate benign from malignant prostate disease more accurately, to identify the clinically important prostate carcinomas and to develop potential targets for new treatment strategies.

A recently discovered prostate-associated biomarker designated prostate-specific membrane antigen (PSMA) may have the properties to meet some or all of these needs. This antigen was first described by Horoszewicz *et al.* (1987) using the mouse 7E11-C5 MAb produced against the LNCaP prostate carcinoma cell line. Immunohistochemical analysis showed that PSMA expression was highly restricted to normal, benign and malignant prostate epithelia (Horoszewicz *et al.*, 1987; Lopes *et al.*, 1990), and the physical nature of the antigen was initially identified in our laboratory as a predominantly 100 kDa membrane-associated glycoprotein (Abdel-Nabi *et al.*, 1992). The cDNA encoding the 100 kDa PSMA has been cloned and the DNA and amino acid sequence determined (Israeli *et al.*, 1993). Clinical trials using an ¹¹¹Indium-labeled conjugated form of MAb 7E11-C5.3 (¹¹¹In CYT-356) to localize metastatic prostate carcinoma and sites of recurrence

following radical prostatectomy (Babaian *et al.*, 1994) were found to be superior to traditional staging and imaging modalities. Phase-I clinical trials have been initiated to evaluate the efficacy of MAb 7E11-C5.3 radionuclide immunoconjugates for treating metastatic prostate cancer (Axelrod *et al.*, 1992). Another possible clinical application using RT-PCR to detect metastatic cells expressing PSMA mRNA in the whole blood of patients has been reported (Israeli *et al.*, 1995).

The PSMA glycoprotein appears to be an important new clinical biomarker of prostate cancer, yet no reports have critically evaluated its tissue specificity and examined whether it is shed into body fluids. Western blot analysis was used in the present study to determine conclusively if the PSMA glycoprotein could be detected with MAb 7E11-C5.3 in body fluids and tissue extracts from normal, benign and malignant prostates and to assess further the specificity of MAb 7E11-C5.3 by evaluating extracts from a variety of non-prostate tissues.

MATERIAL AND METHODS

Cells and reagents

LNCaP and PC3 cells were obtained from the ATCC (Rockville, MD). DU145 cells were kindly provided by D. Mickey (Duke University). Cells were grown in RPMI 1640 supplemented with L-glutamine and 5% FCS (GIBCO-BRL, Gaithersburg, MD). The MAb 7E11-C5.3, purified by protein-A affinity chromatography from murine ascites, was provided by Cytogen (Princeton, NJ). The MAb concentration was determined using a single radial immunodiffusion system (TAGO, Burlingame, CA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

LNCaP xenograft tumors

Male athymic (*nu/nu*) Swiss background nude mice and Fox Chase SCID inbred mice (C.B-17/ICrTac-scld DF), 4-6 weeks old (Taconic Farms, Germantown, NY) were housed in sterilized cages with filter bonnets and given autoclaved laboratory rodent chow (Purina, St. Louis, MO) and filtered tap water *ad libitum*. Mice were given injections of 2.5 mg of cyclophosphamide i.p. 1 day prior to s.c. injections in the left rear flank with 1×10^7 LNCaP cells in exponential growth phase, in 0.2 ml of sterile medium or PBS. Subsequently, LNCaP tumors were propagated by s.c. implantation of tumor fragments aseptically transferred from donor to cyclophosphamide-treated, recipient mice. The same methods were used to grow LNCaP tumors in SCID mice, except SCID mice were not treated with cyclophosphamide.

Tissues and seminal plasma

Frozen human tissues, procured at the time of surgery or at autopsy were obtained from the Virginia Prostate Center tissue bank or through the Southeastern Cooperative Tissue Network (Birmingham, AL). All normal tissues were procured

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at autopsy, and benign and carcinoma tissues were obtained following surgery. Normal semen specimens were obtained from the Andrology Laboratory at the Howard and Georgianna Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, following routine semen analysis. Semen from BPH and prostate carcinoma patients was obtained by masturbation, after informed consent.

Following collection, the semen samples were treated as previously described (Edwards *et al.*, 1981; Pulkkinen *et al.*, 1977), with minor modifications. Briefly, the samples were frozen prior to liquefaction and stored at -70°C , until further analysis. The samples were thawed at room temperature by adding $\frac{1}{2}$ volume dilution buffer (123 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM EDTA and 37 mM Tris, pH 8.0). Pefablock (Boehringer Mannheim, Indianapolis, IN) was added to a final concentration of 0.1 mM and 50 \times protease inhibitor cocktail (0.28 mM ANTIPAIN; 0.75 mM PEPSTATIN; 60 mM EDTA) was added to a final concentration of 1 \times . The samples were centrifuged for 5 min at 25,000g to remove cells or cellular debris and the supernatants, termed seminal plasma, were transferred to Eppendorf tubes and stored at -70°C .

Membrane preparations

LNCaP cells were harvested and pelleted by centrifugation at 1,000g. The pellet was washed once with ice-cold PBS and pelleted again. The pellet was resuspended in hypotonic buffer (1 mM NaCO_3) containing a protease inhibitor cocktail (as described above) and incubated on ice for 30 min; the cells were then lysed by Dounce homogenization. Surgical human tissue or murine xenograft tumors were prepared by mincing the tissue with scissors in 10 ml of hypotonic buffer containing protease inhibitor cocktail and homogenized using a Polytron homogenizer (Brinkmann, Westbury, NY).

The homogenates were centrifuged at 2,000g for 5 min in a Beckman JA20 rotor to pellet whole cells and nuclei. The supernatant was collected and centrifuged at 138,000g for 2 hr in a Beckman Ti50 rotor. The supernatant was discarded and the pellet, representing a crude membrane preparation, was either resuspended in PBS and stored at -70°C or used for antigen purification.

Western blot analysis

Samples were loaded in equal protein concentrations (50 $\mu\text{g}/\text{lane}$ for membrane preps and 200 $\mu\text{g}/\text{lane}$ for seminal plasma) into the lanes of 4–20% gradient SDS-PAGE gels and blotted to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated for 1 hr at 37°C in blocking buffer (5 \times Denhardt's buffer, 1 \times BBS, 0.1% NP-40 and 1.5% BSA). The blocking buffer was removed and the membranes incubated with 7E11-C5.3 (20 $\mu\text{g}/\text{ml}$) for 1 hr at room temperature. The primary antibody was removed and the membranes washed for 10 min \times 3 with wash buffer (30 mM Tris, pH 7.5, 0.5 M NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 0.5 mM DTT); then membranes were incubated with horse anti-mouse horseradish peroxidase-labeled secondary antibody diluted in blocking buffer (1:10,000; Vector, Burlingame, CA) for 1 hr at room temperature. The secondary antibody was removed and the membranes washed for 10 min \times 3 in wash buffer. The blots were developed using the ECL method (Amersham, Arlington Heights, IL) according to the manufacturer's instructions and exposed to X-ray film. Apparent m.w. were calculated on multiple blots using m.w. markers.

Competitive peptide blocking experiments

The competitive blocking studies were carried out like the Western blot experiments described above except the PSMA active peptide N1.19 (Troyer *et al.*, 1995) was added in a 20-fold molar excess over the MAb 7E11-C5.3 during the primary antibody incubation.

PSMA affinity column purification

Membrane preparations were resuspended in solubilization buffer (30 mM Tris, pH 7.5, 0.5 M NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 0.5 mM DTT) and protease inhibitor cocktail (as described above) and incubated for 2 hr at 4°C on a rotator. The solubilized material was centrifuged at 100,000g for 1 hr at 4°C to pellet non-solubilized material. The supernatant was collected, diluted 1:2 with 20 mM Tris, pH 8.0 and loaded onto an Affinica Protein A-7E11-C5 affinity column constructed using the manufacturer's instructions (Schleicher and Schuell, Keene, NH). The column was washed with wash buffer (20 mM Tris, pH 8.0, 0.1% NP-40 and 0.1 mM DTT) and eluted with 2 N NH_4OH , pH 11.0. The eluted fraction was placed in 3,500 m.w. cut-off dialysis tubing (Spectrum, Houston, TX) and dialyzed against 20 mM Tris HCL, pH 4.0, containing 0.1 mM DTT for 2 hr at 4°C . The eluant was then concentrated against polyethylene glycol compound (m.w. 15,000–20,000) to approximately 500 μl . Protein concentrations were estimated using the BCA protein assay following the manufacturer's instructions (Pierce, Rockford IL).

SDS-PAGE purification of PSMA

Affinity column-purified PSMA was loaded into the lanes of an SDS-PAGE mini-gel at 30 $\mu\text{g}/\text{lane}$. One lane of purified PSMA and one m.w. marker lane were stained with Coomassie blue. The remaining PSMA bands were excised using the stained lane as a guide and placed in dialysis tubing (12–14,000 m.w. cut-off; Spectrum) containing 500 μl CAPS buffer (10 mM CAPS, pH 11.0, 0.5 mM DTT) and electroeluted for 2 hr at 12 mAmp in CAPS buffer. The acrylamide bands were removed from the dialysis tubing and the eluted protein was dialyzed against distilled deionized water for 2 hr, then removed from the dialysis tubing and dried in a Savant (Farmingdale, NY) Speed-Vac concentrator.

Analysis of PSMA expression in serum

After informed consent, serum samples from normal donors were collected. Serum from patients with BPH or CaP were obtained from the tissue bank of the Virginia Prostate Center, Eastern Virginia Medical School. Serum samples from 5 non-pregnant normal women, 5 normal men under the age of 40, 5 BPH patients and 5 patients with stage D2 CaP were pooled. Twenty micrograms of affinity-purified PSMA was used to spike 500 μl of the stage D2 pooled serum and incubated for 12 hr at 37°C . An additional 500 μl of the same stage D2 pooled serum representing 29.09 mg of total protein was incubated in solubilization buffer (as described above) for 2 hr at 4°C , applied to a 7E11-C5.3 affinity column, eluted and prepared as described above. Twenty five micrograms of membrane preparations from LNCaP cells and normal prostate tissue; 100 μg of a normal seminal plasma; 400 μg of the spiked D2 serum; 20 μg of affinity-purified stage D2 serum; and 400 μg of non-spiked D2 serum, BPH serum, normal male serum and normal female serum were loaded into the lanes of 2 identical 4–20% SDS-PAGE gels, electrophoresed, blotted and developed for Western blot analysis as described above with either 7E11-C5.3 or an isotype-matched (IgG_1) control antibody.

Two dimensional gel electrophoresis

Two-dimensional experiments were done using the Investigator 2-D Electrophoresis System (Millipore, Marlborough, MA) following the manufacturer's directions. Briefly, 10 μg of affinity-purified antigen was loaded on top of a preparative isoelectric focusing tube gel and overlaid with sample overlay buffer. The tubes were electrophoresed for 17 hr at 1,000 Volts followed by 15 min, at 1,500 Volts. The gels were extruded onto pre-cast 4–20% gradient SDS-PAGE gels and layered with a 1% agarose sticker containing 0.1% bromophenol blue.

The second dimension was run at 16 mA overnight. The gel was removed from the apparatus and blotted to Immobilon-P (Millipore, Bedford, MA) for Western blot analysis. Isoelectric points were estimated by analyzing a tube gel, run at the same time as the sample, which contained only ampholytes. The analytical tube gel was cut into 1 cm sections and incubated in 3 ml double distilled water for 2 hr followed by pH measurement.

RESULTS

PSMA expression in prostate cell lines

Initial Western blots of membrane preparations from LN-CaP xenograft tumors showed a predominant 100 kDa band and 2 higher m.w. species of approximately 180 and 160 kDa. Analysis of membrane extracts from cultured LNCaP cells gave identical results, whereas the prostate cell lines PC3 and DU145 did not express PSMA (Fig. 1 and data not shown). The 160 kDa band and an occasional 70 kDa band were transient and most likely represent breakdown products of the 2 major species. Furthermore, the 180 kDa band could be eliminated by increasing the SDS concentration in the sample buffer (data not shown).

Two-dimensional gel electrophoretic analysis of PSMA

To characterize the PSMA expressed by the LNCaP cell line further, two-dimensional gel electrophoresis was performed using purified PSMA from *in vitro* cultured cell membrane preparations. Surprisingly, identical isoelectric points for both species were observed, with the major spots at isoelectric points of approximately 5.6, 5.7 and 5.8 for both the 100 and 180 kDa bands (Fig. 2). The tailing is consistent with the characteristics of integral membrane proteins. These results suggest that the 2 species are highly similar in their biochemical nature.

Comparison of PSMA100 and PSMA180

The fact that identical isoelectric points were observed indicated that PSMA may exist in a dimer form. To look at this relationship more closely, affinity column-purified PSMA was separated by SDS-PAGE. The 2 m.w. species of 100 and 180 kDa are clearly evident (Fig. 3, lane 1) along with some smaller bands, which most likely represent breakdown products. The 2 major bands were excised, electroeluted, reapplied to an SDS gel and immunoblotted with MAb 7E11-C5.3. As shown in Figure 3, lane 2, the majority of the 100 kDa band remained at the appropriate m.w. However, a minority of this band migrated at the higher m.w. of 180 kDa. At the same time, the

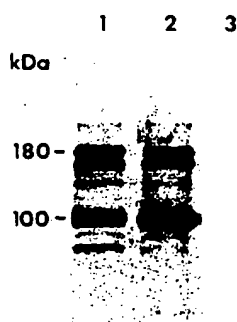


FIGURE 1 - Western blot analysis of 50 µg of membrane preparations from LNCaP nude mouse tumors (lane 1), cultured LNCaP cells (lane 2) and PC3 cells (lane 3). The 100 and 180 kDa bands are present in the LNCaP extracts but absent from PC3 membrane preparations.

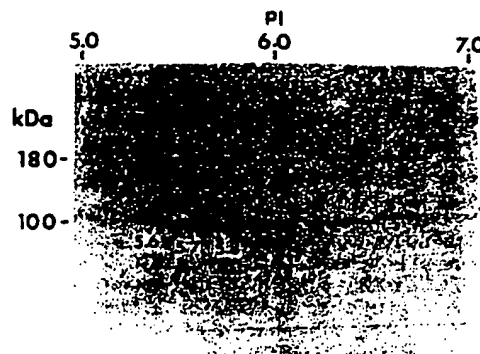


FIGURE 2 - Two-dimensional gel electrophoresis of affinity-purified PSMA. Ten micrograms of purified PSMA was analyzed as described in Material and Methods. Arrows indicate the isoelectric points of 5.6, 5.7 and 5.8 showing identical spots for both the 100 and 180 kDa species.

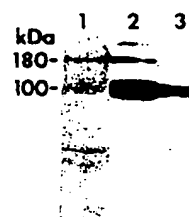


FIGURE 3 - Purified PSMA was separated on an SDS-PAGE gel and stained with Coomassie blue (lane 1); the predominant bands of 100 and 180 kDa and some smaller breakdown products are shown. The individual bands were excised and purified by electroelution from the gel slices. Gel-purified PSMA100 (lane 2) and PSMA180 (lane 3) were then reanalyzed by Western blot, which showed the majority of both species migrating at 100 kDa, indicating that PSMA180 is a dimer of PSMA100.

majority of the 180 kDa protein migrated faster at 100 kDa with a minority at 180 kDa (Fig. 3, lane 3). Increased concentrations of mercaptoethanol added to the loading buffer was not sufficient to reduce the 180 kDa band to 100 kDa in LNCaP membrane extracts, whereas increased SDS was able to denature the 180 kDa band totally to 100 kDa (data not shown). Additionally, trypsin digestion of the 2 bands followed by analysis on a silver-stained SDS-PAGE gel strongly indicated that the 2 bands were identical (data not shown). These data suggest that the 180 kDa species may be a dimer of the 100 kDa band or another unidentified molecule that may not be completely denatured by the normal concentration of SDS in the loading buffer used for the SDS-PAGE gel analysis.

Detection of PSMA in prostate tissue

Membrane preparations of pathology confirmed normal, benign and malignant prostate tissues showed a Western blot banding pattern similar to that seen in LNCaP cell preparations except the migration of both the 100 and 180 kDa species had slightly slower mobilities of 120 and 200 kDa, respectively (Fig. 4a,b, arrows). This expression of PSMA was observed in cerebral cortex and salivary gland membrane extracts (Fig. 5, lanes 4 and 5). The size of PSMA expressed by the cerebral cortex was similar to that seen in LNCaP cell line extracts (approximately 100 kDa) whereas the size of PSMA detected in the salivary gland was similar to that seen in prostate

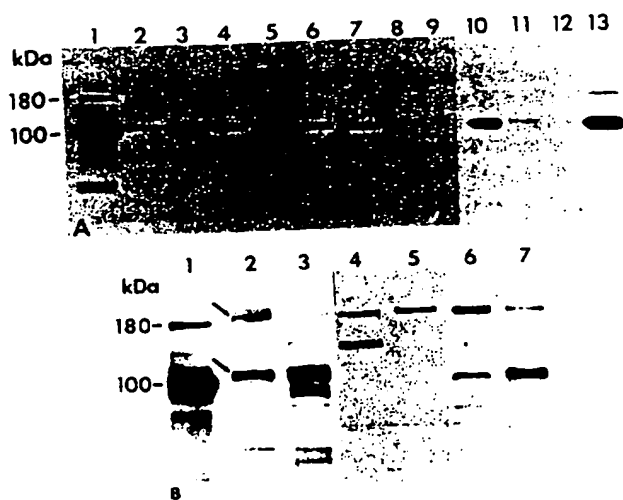


FIGURE 4 – Detection of PSMA in prostate tissue. (a) Western blot of 50 μ g of LNCaP membrane preparation (lane 1), normal prostate tissue extracts (lanes 2–5), BPH tissue extracts (lanes 6–9) and CaP tissue extracts (lanes 10–13). (b) Western blot analysis of prostate extracts showing some of the heterogeneity seen in some samples—50 μ g of LNCaP membrane preparation (lane 1), normal prostate tissue extracts (lanes 2 and 3), BPH tissue extracts (lanes 4 and 5) and CaP tissue extracts (lanes 6 and 7). Arrows indicate the shift in the size of the bands from the 100 and 180 kDa of LNCaP PSMA to the approximately 120 and 200 kDa of the PSMA seen in tissue extracts.

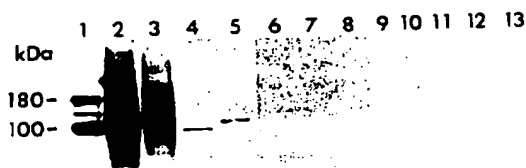


FIGURE 5 – Detection of PSMA in normal non-prostate tissue extracts. Western blot of 50 μ g of membrane preparations from LNCaP cells (lane 1), small intestine (lanes 2 and 3), cerebral cortex (lane 4), salivary gland (lane 5), skeletal muscle (lane 6), cardiac muscle (lane 7), colon (lane 8), breast (lane 9), lung (lane 10), ovary (lane 11), kidney (lane 12) and liver (lane 13). Lane 3 represents 25 μ g of the same small intestine shown in lane 2. The small intestine (lanes 2 and 3) shows a smearing with no detectable bands, similar to what is seen in prostate tissues. The PSMA in the cerebral cortex (lane 4) is similar in size to the 100 kDa species of LNCaP cells, whereas the PSMA expressed in salivary gland (lane 5) is similar to that seen in prostate tissue.

tissue extracts (approximately 120 kDa). The PSMA glycoprotein was not expressed in the majority of non-prostate tissues examined including normal skeletal and cardiac muscle, colon, breast, lung, ovary, kidney and liver tissues (Fig. 5, lanes 5–12) or a variety of non-prostate malignancies including colon, lung, bladder, liver and breast adenocarcinomas (Fig. 6, lanes 2–6).

7E11-C5.3 reactive bands could be competitively blocked with PSMA peptides

The peptide epitope for MAb 7E11-C5.3 on the PSMA glycoprotein has been determined to be located at the amino terminal end of the polypeptide backbone (Troyer *et al.*, 1995).

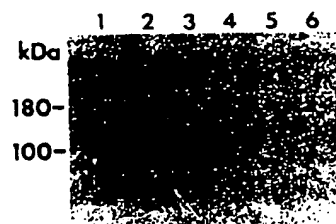


FIGURE 6 – Detection of PSMA in non-prostate malignancies. Western blot analysis of 50 μ g of membrane preparations from LNCaP cells (lane 1), colon (lane 2), lung (lane 3), bladder (lane 4), liver (lane 5) and breast adenocarcinoma (lane 6) showing the typical 100 and 180 kDa bands in the LNCaP cells but no expression in other malignant tissues.

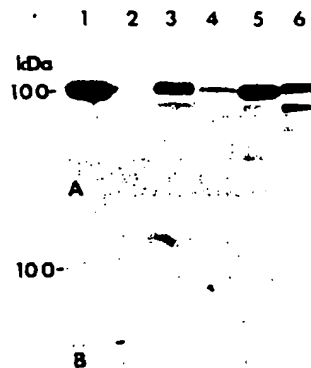


FIGURE 7 – Western blot competitive inhibition of MAb 7E11-C5.3 with the PSMA peptide N1.19. (a) Membrane extracts from LNCaP (lane 1), PC3 (lane 2), normal prostate (lane 3), benign prostate (lane 4), prostate carcinoma (lane 5) and a seminal plasma from a prostate carcinoma patient probed with MAb 7E11-C5.3. (b) The identical blot probed with MAb 7E11-C5.3 plus a 20 M excess of the PSMA N1.19 peptide. The N1.19 peptide is clearly able to abrogate MAb 7E11-C5.3 binding to its target antigen on prostate cell lines, tissues and seminal plasma.

The reactive peptide N1.19, representing the first 19 amino acids of PSMA, was used to block MAb 7E11-C5.3 reactivity competitively in immunoblot assays. The reactivity to LNCaP cell line extracts, prostate tissue extracts, and seminal plasma (Fig. 7a) could be totally eliminated by incubating the antibody with a 20-fold molar excess of the N1.19 peptide (Fig. 7b).

PSMA was not detected in the serum of prostate carcinoma patients

Since other prostate markers are detected in significant concentrations in serum, we wanted to determine if PSMA could also be detected in serum by Western blot analysis. Figure 8a shows the analysis of serum by Western blotting with MAb 7E11-C5.3. When these blots were exposed for the standard amount of time (1 to 2 min), no bands were evident, but when the blots were exposed for more than 10 min the pattern in Figure 8 was observed. Lanes 1–3 in Figure 8a represent LNCaP, normal prostate tissue membrane extracts and normal seminal plasma, respectively. Note the difference in size of the PSMA expressed in LNCaP (Fig. 8a, lane 1) compared with the tissue and seminal plasma (Fig. 8a, lanes 2 and 3). The size of PSMA shed into serum would be expected to be similar to the size of PSMA observed in tissue extracts and seminal plasma. Sera from 5 stage D2 CaP patients were pooled and analyzed in several ways. First, the serum was

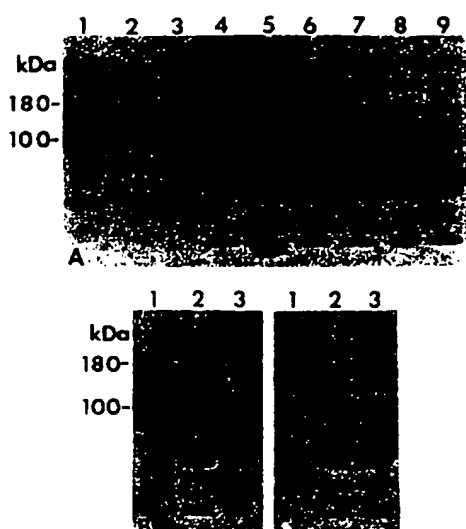


FIGURE 8 – Detection of PSMA in serum. (a) Western blot, using uE11-C5.3, of 25 μ g of an LNCaP membrane extract (lane 1) and normal prostate (lane 2) membrane extracts, 100 μ g of normal seminal plasma (lane 3), 400 μ g of spiked pooled stage D2 serum (lane 4), 20 μ g of affinity-purified stage D2 serum (lane 5), 400 μ g of pooled serum from stage D2 CaP (lane 6), BPH (lane 7), normal man (lane 8) and normal women (lane 9). (b) Western blot of 25 μ g of an LNCaP membrane extract (lane 1), 400 μ g of pooled D2 serum (lane 2) and 800 μ g of pooled D2 serum blotted with MAb 7E11-C5.3. (c) The identical blot probed with MAb 7E11-C5.2 plus N1.19 PSMA peptide. No PSMA was detected by affinity chromatography of the pooled D2 serum (a, lane 5), and whereas bands are present in the pooled sera, the same bands are present in the pooled normal male and female serum. The bands in the LNCaP extract were eliminated by competition with the N1.19 peptide whereas the bands in the pooled serum remained.

spiked with affinity-purified LNCaP PSMA and incubated for 12 hr at 37°C prior to analysis by Western blot (Fig. 8a, lane 4). The PSMA in the spiked serum was at the expected m.w. of 100 and 180 kDa, like the PSMA seen in LNCaP extracts, along with a larger band of unknown composition. Second, 29 mg of the pooled stage D2 serum was solubilized, passed through a MAb 7E11-C5.3 affinity column, washed and eluted from the column to remove the large concentrations of albumin and other proteins that make Western blot analysis of serum difficult. No PSMA was detected in the affinity column eluant although a significant amount of serum proteins remained, particularly bands migrating at the expected m.w. of the heavy and light immunoglobulin chains, which may have adhered to the protein-A used to construct the affinity column (Fig. 8a, lane 5). Third, 400 μ g of the same stage D2 serum and 400 μ g of a pooled BPH, a pooled normal male and a pooled normal female serum (Fig. 8a, lanes 6–9) were analyzed. Several bands were present following overexposure in all the sera including the normal female serum but none at the expected m.w. of 120 kDa.

The bands present in the pooled stage D2 serum did appear to be overexpressed compared with the BPH and normal sera. The presence of identical bands in the pooled normal female serum suggested that the bands seen on this blot were non-specific. To determine if this were the case, the D2 pooled serum was subjected to SDS-PAGE gel electrophoresis in duplicate along with an LNCaP membrane extract. One of the blots was probed with MAb 7E11-C5.3 and the duplicate blot was probed with MAb 7E11-C5.3 containing the N1.19 PSMA

peptide. Figure 8b shows the banding pattern for the LNCaP extract (Fig. 8b, lane 1) and 400 and 800 μ g of the pooled D2 serum (Fig. 8b, lanes 2 and 3, respectively). There is a reactive band at the approximate m.w. of 100 kDa in this pooled serum that could be interpreted to be PSMA. However, while all of the reactive bands seen in the LNCaP membrane extract were eliminated by the N1.19 peptide competition (Fig. 8c, lane 1) none of the bands in the serum were competed out (Fig. 8c, lanes 2 and 3). An identical pattern could also be produced if the pooled serum blot was probed with only the secondary antibody or with an isotype-matched IgG₁ control antibody in place of MAb 7E11-C5.3 (data not shown).

Detection of PSMA in seminal plasma

Since PSMA is a product of the prostate glandular epithelium, it was of interest to determine whether it could be detected in seminal plasma. Western blot analysis of seminal plasma obtained from normal (NSP), BPH (BSP) and prostate carcinoma patients (CaSP) showed that PSMA was readily detectable in seminal plasma with expression of the 120 kDa band and occasionally the 200 kDa band, similar to the pattern seen in tissue extracts (Fig. 9). NSP showed the most consistent expression of PSMA, with little variation from sample to sample, demonstrating the 120 kDa band and often an 80 kDa band (Fig. 9, lanes 2–5). BSP, like the BPH tissue extracts, exhibited variable expression of PSMA ranging from very low to overexpression (Fig. 9, lanes 6–9). The majority of the CaSP samples were similar in banding pattern to NSP, although one sample showed only the 180 kDa band. While it appears that the concentration of PSMA in CaSP is lower than in NSP, the total protein of these samples was significantly higher than in NSP or BSP. In fact, when equal volumes of samples instead of equal protein concentrations were used for the immunoblots, the intensities of the CaSP bands were comparable to those seen in the normal samples (data not shown).

DISCUSSION

PSMA is a new prostate biomarker that appears to be overexpressed in poorly differentiated and metastatic prostate carcinomas (Wright *et al.*, 1995). Antibody-radionuclide conjugates have been successfully used to localize metastatic disease *in vivo* (Babaian *et al.*, 1994) and to treat human prostate

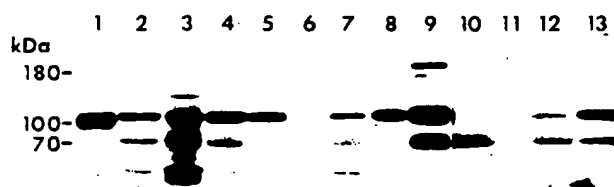


FIGURE 9 – Detection of PSMA in seminal plasma. Western blot of 50 μ g LNCaP membrane preparation (lane 1) and equal protein concentrations (200 μ g/lane) of normal seminal plasma (lanes 2–5), BPH seminal plasma (lanes 6–9) and CaP seminal plasma. Lanes 1–9 represent an exposure time of 2 min and lanes 10–13 represent an exposure of 5 min. Note the slightly larger m.w. of PSMA in the seminal plasma compared with LNCaP extracts. Normal seminal plasma shows a consistent expression of PSMA whereas BPH seminal plasma is quite variable. The expression of PSMA in CaP seminal plasma appears to be lower, which may result from the much higher protein concentrations seen in CaP compared with normal or BPH seminal plasma.

tumors in nude mice (Axelrod *et al.*, 1992). These reports suggest that PSMA may have important diagnostic and therapeutic applications. The present study provides information on the presence and molecular size characteristics of PSMA in body fluids and tissue extracts from normal donors and from patients with benign and malignant tumors.

The finding of 120 and 200 kDa bands in prostate tissue extracts was in contrast to the 100 and 180 kDa bands found in membrane extracts and purified PSMA preparations from the LNCaP prostate carcinoma cell line. This slower mobility of PSMA in tissue extracts and seminal plasma may result from a post-translational modification or an alternative splicing event. The fact that normal, BPH and CaP tissue extracts exhibited the same size components (either the 120 kDa band, the 200 kDa band or both) indicates that the PSMA glycoprotein recognized by MAb 7E11-C5.3 is synthesized identically in normal and pathological prostate tissues. Additionally, the detection of PSMA in normal prostate tissue did not appear to be influenced by the significant time period between death and the collection of tissue during autopsy since the normal prostatic tissue (autopsy specimens) and CaP tissue (surgical specimens) showed similar results.

The occasional higher m.w. species was most often seen when there was a large amount of the smaller band, suggesting that a strong relationship may exist between the low and high m.w. components. Although it is possible that the 100/120 kDa species was a breakdown product of the 180/200 kDa band or that the 180/200 kDa component represented a precursor molecule of the 100/120 kDa band, one- and two-dimensional electrophoretic analysis of purified PSMA from LNCaP extracts demonstrated that the 180 kDa species may be a dimer of the 100 kDa component. The findings that the 100 kDa band may redimerize following purification, a phenomenon that has been reported to occur with other proteins (Huth *et al.*, 1993), that the dimer was observed under denaturing and reducing conditions (Reiser *et al.*, 1992) and that the PSMA cDNA clone encodes for a 100 kDa glycoprotein (Israeli *et al.*, 1994) support the hypothesis that the 180/200 kDa component is most likely a dimer of 2 PSMA 100/120 kDa molecules.

PSMA was not found in extracts of other prostate cell lines, supporting previous reports (Horoszewicz *et al.*, 1987; Israeli *et al.*, 1994). Horoszewicz *et al.* (1987) reported the finding of PSMA in frozen sections of proximal tubules of normal kidneys, and Lopes *et al.* (1990) described PSMA expression in cardiac and skeletal muscle by immunoperoxidase staining. The same immunostaining reactivity was observed in formalin-fixed, paraffin-embedded sections of these normal tissues (data not shown). However, in the present study, PSMA was not detected in extracts of these same normal tissues by Western blot analysis, which suggests that the immunoperoxidase staining was non-specific. This conclusion is consistent with the fact that 7E11-C5.3 antibody-isotope conjugates did not localize to skeletal muscle in mice (Lopes *et al.*, 1990) or in monkeys (D. Lopes, Princeton, NJ, personal communication). The detection of PSMA in extracts of normal cerebral cortex of the brain, normal salivary gland and normal small intestine correlates with the finding of PSMA mRNA in brain and salivary gland and no mRNA in other normal tissues, including skeletal muscle (Israeli *et al.*, 1994). It is of interest to note that PSA has been found to be present in breast, colon, ovarian, parotid, kidney and liver tumors, normal breast, amniotic fluid, breast milk (Levesque *et al.*, 1995), normal salivary gland (van Krieken, 1994) and normal endometrium (Clements and Mukhtar, 1994). These results suggest that PSA as well can no longer be regarded as a specific biomarker of the prostate gland. In spite of the detection of PSMA in these tissues by immunoblotting, it has not been detected by immunostaining of frozen sections (Horoszewicz *et al.*, 1987; Lopes *et al.*, 1990)

or paraffin-embedded sections (data not shown) of normal brain, salivary gland and small intestine. This disparity may be the result of masked antigenic epitopes in the tissue sections or the fact that expression was below the detection limits of the immunohistochemistry assay. By using more sensitive assays, PSMA, like PSA, may eventually be found to be present in a variety of human tissues. The possibility that PSA may have a broader function than its association with semen liquefaction (Levesque *et al.*, 1995) points to the importance of determining the function of PSMA.

The finding that different species of PSMA were detected in brain (100 kDa) and salivary gland (120 kDa) supports the hypothesis that the differences seen between PSMA from LNCaP cells and prostate tissue extracts may result from a change in post-translational modification or a splicing variant and are not artifacts of *in vitro* cell culture. The high m.w. smearing and multiple banding pattern of the immunoblot of the small intestine formed a pattern markedly different from that observed in LNCaP cell extracts and prostate and non-prostate extracts. The banding pattern was suggestive of a heavily glycosylated glycoprotein or mucin. It is quite possible that MAb 7E11-C5.3 binds to an epitope other than PSMA in the small intestine, or such a pattern may be due to non-specific binding of the antibody. Since the secondary antibody (used without the primary MAb 7E11-C5.3) did not bind blots of small intestine, it appears likely that MAb 7E11-C5.3 specifically bound to a component in this extract. PSMA may be glycosylated differently in the intestine, thereby resulting in the different banding pattern.

The detection of PSMA in seminal plasma but not serum by immunoblot analysis was surprising since previous reports suggested that PSMA could be detected in serum by an immunoassay (Horoszewicz *et al.*, 1987) and Western blot (Rochon *et al.*, 1994). The analysis of serum in the present study resulted in multiple bands similar to those reported by Rochon *et al.* (1994). They identified a band of 116 kDa as the PSMA component. In the present study a band between 100 and 120 kDa was observed, and although it did appear that the bands in pooled stage D2 serum were slightly overexpressed compared with normal and BPH serum, the same banding pattern was observed in both female and male serum, suggesting that these bands were the results of non-specific binding with either the primary or secondary antibody. When the PSMA peptide (N1.19) containing the MAb 7E11-C5.3 epitope (Troyer *et al.*, 1995) was incubated with the antibody prior to use as a probe, the PSMA bands in the LNCaP cell extract were eliminated, whereas all the bands in the serum samples remained. These results clearly suggest that the bands observed in serum are non-specific, probably the result of non-specific binding by the secondary antibody. Since Rochon *et al.* (1994) did not compete the reactivity with purified PSMA or show results using only the secondary antibody, the 116 kDa band they reported as being PSMA is most likely a serum protein reacting with the secondary antibody. In fact, we could demonstrate an identical banding pattern in serum using only the secondary antibody for the immunoblot (data not shown). These results and conclusions do not negate the possibility that PSMA is shed into serum. The failure to detect PSMA by Western blot may have been the result of degradation or masking of the antigenic epitope, although purified PSMA or specific PSMA peptides added to serum were found to be stable and of the correct m.w. when immunoblotted. Alternatively, the concentration of PSMA in serum may have been too low to be detected by Western blotting. However, PSMA was not detected following the enrichment (affinity purification) of serum over 200-fold.

The 120 or the 200 kDa band (or both) PSMA components were found to be present in seminal plasma from normal

donors and from patients with benign or malignant prostate tumors. The PSMA detected in seminal plasma from normal males and prostate carcinoma patients showed a consistent banding pattern, whereas the banding pattern in seminal plasma from BPH patients varied greatly. This variation is consistent with the observed reduction in PSMA mRNA (Israeli *et al.*, 1994) and PSMA antigen expression (Wright *et al.*, 1995) in BPH specimens. The often low, absent and variable expression of the 120 kDa band in the BPH specimens may have resulted from splicing variants or post-translational modifications.

Previously we described the mapping of the Mab 7E11-C5.3 epitope on PSMA (Troyer *et al.*, 1995) and analyzed PSMA in prostate tissue immunohistochemically (Wright *et al.*, 1995). In the present study we have shown that PSMA exists in tissues and seminal plasma as a predominant 120 kDa band; by contrast, PSMA is found as a 100 kDa glycoprotein in extracts of LNCaP cells. Occasionally, a dimer form of PSMA having a m.w. of 180 (LNCaP cell extracts) or 200 kDa (tissue extracts, seminal plasma) is observed. The finding of PSMA in seminal plasma, coupled with the overexpression of PSMA observed in poorly differentiated and metastatic prostate tissues (Wright *et al.*, 1995), suggests that measurement of PSMA concentrations

in seminal plasma might be used to predict and monitor tumor progression. Although Western blot analysis failed to detect PSMA in serum, further studies will be required to determine if any form of PSMA is shed in serum. The production of second generation antibodies against different antigenic epitopes other than the one recognized by Mab 7E11-C5.3 may be required to develop a highly sensitive immunoassay to determine if PSMA is or is not present in serum. Finally, the finding of PSMA in extracts of brain, salivary gland and small intestine raises certain issues regarding the safety of using Mab 7E11-C5.3 immunoconjugates for diagnostic imaging and especially antibody-targeted therapy. These cross-reactivities, however, should not dampen efforts to understand fully the function and clinical potential this novel biomarker may have in the diagnosis and therapy of prostate cancer.

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REFERENCES

- ABDEL-NABI, H., WRIGHT, G.L., JR., GULFO, J.V., PETRYLAK, D.P., NEAL, C.B., TEXTER, J.E., BEGHUN, F.P., TYSON, I., NEAL, A., MITCHELL, E., PURNELL, G. and HARWOOD, S.J., Monoclonal antibodies and radioimmunoconjugates in the diagnosis and treatment of prostate cancer. *Semin. Urol.*, 10, 45-54 (1992).
- AXELROD, H.R., GILMAN, S.C., D'ALEO, C.J., PETRYLAK, D., REUTER, V., GULFO, J.V., SAAD, A., CORDON-CARDO, C. and SCHER, H.I., Preclinical results and human immunohistochemical studies with 90Y-CYT-356: a new prostate cancer therapeutic agent. *J. Urol.*, 147, 361A (Abs. 596) (1992).
- BABAIAN, R.J., SAYER, J., PODOLOFF, D.A., STEELHAMMER, L.C., BHADKAMKER, V.A. and GULFO, J.V., Radioimmunosintigraphy of pelvic lymph nodes with ¹¹¹indium-labeled monoclonal antibody CYT-356. *J. Urol.*, 152, 1952-1955 (1994).
- CLEMENTS, J. and MUKHTAR, A., Glandular kallikrins and prostate specific antigen are expressed in the human endometrium. *J. clin. Endocrinol. Metab.*, 78, 1536-1539 (1994).
- CUPP, M.R. and OESTERLING, J.E., Prostate specific antigen, digital rectal exam, and transrectal ultrasonography: their roles in diagnosing early prostate cancer. *Mayo. Clin. Proc.*, 68, 297-306 (1993).
- EDWARDS, J.J., TOLLAKSEN, S.L. and ANDERSON, N.G., Two-dimensional mapping of human seminal fluid. *Clin. Chem.*, 27, 1335-1340 (1981).
- HOROSZEWICZ, J.S., KAWINSKI, E. and MURPHY, G.P., Monoclonal antibodies to a new antigenic marker in epithelial prostate cells and serum of prostatic cancer patients. *Anticancer Res.*, 7, 927-936 (1987).
- HUTH, J.R., PERINI, F., LOCKRIDGE, O., BEDOWS, E. and RUDDON, R.W., Protein folding and assembly in vitro parallel intracellular folding and assembly. *J. biol. Chem.*, 268, 16472-16482 (1993).
- ISRAELI, R.S., MILLER, W.H., JR., SU, S.L., SAMADI, D.S., POWELL, C.T., HESTON, W.D.W., WISE, G.J. and FAIR, W.R., Sensitive detection of prostatic hematogenous tumor cell dissemination using prostate specific antigen and prostate specific membrane-derived primers in the polymerase chain reaction. *J. Urol.*, 153, 573-577 (1995).
- ISRAELI, R.S., POWELL, C.T., CORR, J.G., FAIR, W.R. and HESTON, D.W., Expression of the prostate-specific membrane antigen. *Cancer Res.*, 54, 1807-1811 (1994).
- ISRAELI, R.S., POWELL, C.T., FAIR, W.R. and HESTON, D.W., Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.*, 53, 227-230 (1993).
- LEVESQUE, M., HU, H., D'COSTA, M. and DIAMANDIS, E.P., Prostate-specific antigen expression by various tumors. *J. clin. Lab. Anal.*, 9, 123-128 (1995).
- LOPES, A.D., DAVIS, W.L., ROSENSTRAUS, M.J., UVEGES, A.J. and GILMAN, S.C., Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from anti-prostate monoclonal antibody 7E11-C5. *Cancer Res.*, 50, 6423-6429 (1990).
- PULKKINEN, P., SINERVIRTA, R. and JANNE, J., Mechanism of action of oxidized polyamines on the metabolism of human spermatozoa. *J. Reprod. Fertil.*, 51, 399-404 (1977).
- REISER, K., MCCORMICK, R.J. and RUCKER, R.B., Enzymatic and non-enzymatic cross-linking of collagen and elastin. *FASEB. J.*, 6, 2439-2449 (1992).
- ROCHON, Y.P., HOROSZEWICZ, J.S., BOYNTON, A.L., HOLMES, E.H., BARREN, R.J., III, ERICKSON, S.J., KENNY, G.M. and MURPHY, G.P., Western blot assay for prostate-specific membrane antigen in serum of prostate cancer patients. *Prostate*, 25, 219-223 (1994).
- TROYER, J.K., FENG, Q., BECKETT, M.L. and WRIGHT, JR., G.L., Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate specific membrane antigen. *Urol. Oncol.*, 1, 29-37 (1995).
- VAN KRIEKEN, J.H.J.M., Prostate marker immunoreactivity in salivary gland neoplasms. *Amer. J. surg. Pathol.*, 17, 410-414 (1994).
- WINGO, P.A., TONG, T. and BOLDEN, S., Cancer statistics, 1995. *CA*, 45, 8-30 (1995).
- WRIGHT, G.L., JR., HALEY, C., BECKETT, M.L. and STEELHAMMER, P.F., Expression of prostate specific membrane antigen (PSMA) in normal, benign, and malignant prostate tissues. *Urol. Oncol.*, 1, 18-28 (1995).

Location of Prostate-Specific Membrane Antigen in the LNCaP Prostate Carcinoma Cell Line

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BACKGROUND. Prostate-specific membrane antigen (PSMA) is a novel prostate biomarker overexpressed in poorly differentiated and metastatic prostate carcinomas and apparently upregulated following hormone-ablation therapy. PSMA appears to be a satisfactory target for antibody-directed imaging of prostate carcinomas despite the recent finding that the antigenic epitope recognized by monoclonal antibody (MAb) 7E11-C5 is found in the cytoplasmic domain of this transmembrane glycoprotein [Troyer et al.: *Urol Oncol* 1:29-37, 1995]. This finding prompted the present investigation to precisely define the cellular localization of PSMA in the LNCaP prostate carcinoma cell line, the line used to generate MAb 7E11-C5.

METHODS. Subcellular fractionation, immunofluorescence and immunoperoxidase staining of live and fixed cells, and immunoelectron microscopy were used to determine the localization of PSMA in LNCaP cells.

RESULTS. PSMA was found to be localized at the inner face of the plasma membrane as well as being associated with mitochondria. Staining of LNCaP cells, treated by serum starvation followed by serum stimulation, showed no changes in the typical cytoplasmic staining pattern.

CONCLUSIONS. The data suggest that the PSMA target epitope for antibody-directed imaging with MAb 7E11-C5 only becomes accessible upon apoptosis or necrosis. This further suggests that antibodies directed at the extracellular domain may enhance the sensitivity of antibody-directed imaging and therapy of prostate carcinomas by recognizing surface epitopes of PSMA on living cancer cells. *Prostate* 30:232-242, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: tumor; biomarker; cancer; tumor antigen; PSMA

INTRODUCTION

Prostate cancer is the most commonly diagnosed adenocarcinoma and the second most common cause of cancer deaths in men in the United States [1]. The number of deaths attributed to prostate cancer is increasing at a rate of approximately 8% a year [1], which means that between 1995 and the year 2000, 2 million males will be diagnosed and of these approximately 300,000 will have died in just a 5-year time span. Therapy options for prostate cancer have not improved over the past decade and remain limited. Additionally, few model systems exist which allow for the study of novel therapeutic modalities. Therefore, in order to advance the treatment options for

prostate cancer, novel strategies for diagnosis and therapy will be needed to improve the life expectancy of patients diagnosed with this disease.

The use of monoclonal antibody (MAb)-directed imaging and therapy has shown great promise for improving the survival of prostate cancer patients. However, improvements such as increased sensitivity and specificity must be realized before many of these antibody-directed techniques can be routinely utilized for clinical applications. These improvements

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are entirely dependent on an in-depth understanding of the MAb, the antigenic epitope recognized by the antibody, and the molecule which carries the epitope. With this knowledge in hand, appropriate modifications may be made to improve existing applications.

The prostate-specific membrane antigen (PSMA) is recognized by the MAb 7E11-C5.3 which was first described by Horoszewicz et al. in 1987 [2]. It appears to be overexpressed in poorly differentiated and metastatic prostate carcinomas [3] and antibody-radionucleotide conjugates have been successfully used to localize metastatic disease in vivo [4,5] and to treat human prostate tumors in nude mice [6]. These reports suggest PSMA may have promise as an important new diagnostic and therapeutic tool for prostate cancer. Like many clinical strategies, the sensitivity of MAb 7E11-C5.3 conjugates for use in the diagnosis or treatment of prostate cancer may depend on the accessibility of the 7E11-C5.3 epitope within a tumor mass. Previous reports have indicated that the MAb 7E11-C5.3 epitope on PSMA is located in the proposed intracellular domain of the PSMA molecule [7] which would seem to hinder the ability of MAb 7E11-C5.3 to bind to PSMA in intact cells. In the present study, the definitive immunolocalization of MAb 7E11-C5.3 in LNCaP cells has been determined by subcellular fractionation, and immunofluorescence and immunoelectron microscopy.

MATERIALS AND METHODS

Monoclonal and Polyclonal Antibodies

The MAb 7E11-C5.3, purified by protein-A affinity chromatography from murine ascites, was provided by Cytogen Corporation (Princeton, NJ). The MAb concentration was determined using a single radial immunodiffusion system (TAGO, Burlingame, CA). MAbs OKT-9 (Ortho Immunology Systems, Raritan, NJ), MU213 (BioGenex, San Ramon, CA), and proliferating cell nuclear antigen (PCNA; DAKO Corporation, Carpinteria, CA) were purchased and utilized according to the manufacturers' recommendations.

Tissue Culture

LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 medium supplemented with L-glutamine and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD) at 37°C and 5% carbon dioxide.

Mechanical Subcellular Fractionation

The method employed here was modeled after several studies found in the literature [8-10]. Briefly, cultured LNCaP cells (2×10^6) were harvested and

lysed in a hypotonic medium (1 mM NaHCO₃), then dounce homogenized with 50 up and down strokes. It was imperative that the majority of the cells were lysed since the initial centrifugation steps would pellet out unbroken cells as well as nuclei. Therefore, the homogenization was monitored by trypan blue staining and the subsequent steps were not performed until >99% of the cells were lysed. The homogenate was centrifuged at 500g for 5 min at 4°C and the pellet resuspended in buffer containing 16% sucrose then underlayered with 20% sucrose and centrifuged in a swinging bucket rotor at 150,000g for 60 min at 4°C. The nuclei were pelleted to the bottom of the 20% sucrose while other membrane components remained at the interface of the two layers. The supernatant was removed by aspiration and the nuclear pellet resuspended in 16% sucrose and centrifuged again to remove any residual membranes since it has been shown to be difficult to obtain totally pure nuclei [10]. The supernatant from the 500g spin was centrifuged at 10,000g for 15 min at 4°C. The pellet from this step represented the heavy membrane fraction (HM) containing predominately the rough endoplasmic reticulum (ER), the Golgi apparatus, and mitochondria. The supernatant was centrifuged at 150,000g for 60 min at 4°C. The pellet from this step was the light membrane fraction (LM) containing smooth endoplasmic reticulum, plasma membrane, and any vesicular membranes while the supernatant represented the soluble cytoplasmic fraction (C).

The fractions obtained from this fractionation method were analyzed by Western blot and enzyme-linked immunosorbent assay (ELISA) for the presence of PSMA. Control antibodies, used as markers for mitochondria (MU213), plasma membrane (OKT-9), and nucleus (α PCNA), were utilized to verify that the partitioning was efficient.

Living LNCaP Immunofluorescence Staining

LNCaP cells were grown to approximately 50% confluency on collagen-coated chambered slides (Nunc, Naperville, IL). The medium was removed by aspiration and the cells were washed twice with phosphate-buffered saline (PBS; 136 mM NaCl, 1.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.9 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4) warmed to 37°C. Blocking serum (10% goat serum in PBS) was added to the chambers and incubated at room temperature for 1 hr then removed by aspiration. The primary antibody (7E11-C5.3 at 20 μ g/ml, OKT-9 at 15 μ g/ml, PSA-5 at 1 μ g/ml, or IgG1 isotype-matched control at 10 μ g/ml) was added to the cells and incubated at room temperature in a humid chamber for 1 hr. The cells were washed twice with PBS followed by the

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addition of secondary antibody (fluorescein isothiocyanate [FITC]-labeled goat F(ab') α mouse antibody with Evan's blue counter stain [Baxter Healthcare Corporation, West Sacramento, CA]) and incubated at room temperature for 1 hr. The cells were washed twice with PBS then visualized with a fluorescence microscope. Duplicate specimens were prepared for each antibody tested in the above experiment and all subsequent microscopic techniques used.

Fixed LNCaP Cell Immunofluorescence Staining

LNCaP cells were grown to approximately 50% confluency on chambered slides as described above. The cells were fixed for 20 min in 10% buffered formalin and rinsed twice with PBS. The cells were permeabilized briefly in 0.1% Triton-X100 in PBS for 5 min, washed twice with PBS, then stained as described above using 7E11-C5.3, OKT-9, PSA-5, and the IgG isotype-matched negative control antibody.

Immunoperoxidase Staining

The immunoperoxidase staining was carried out exactly as described above for the fixed immunofluorescence staining, except that the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used with a peroxidase-labeled horse α mouse antibody. The positive staining was visualized by the addition of a color substrate (diaminobenzidine [DAB]) for 10 min, then rinsed twice with PBS and counterstained with Mayer's hematoxylin for 5 min. Cytospin preparations were also made of LNCaP cells by first removing adherent cells from culture flasks using PBS-ethylenediaminetetraacetic acid (EDTA). Cells were washed twice in PBS and then resuspended in 10 ml PBS. Cytospin cups were loaded with 200 μ l of cell suspension and spun at 400 rpm for 5 min. Slides were air dried and fixed for 20 min in 10% buffered formalin, followed by 5 min in 70% ethanol. The cytospin slides were then stained as described above for chambered slides.

Immunoelectron Microscopy

To definitively determine the localization of the PSMA glycoprotein, a method for immunoelectron microscopy of PSMA was designed from previously reported studies in the literature [11-13]. LNCaP cells were grown to confluency on plastic coverslips. The coverslips were fixed in half-strength Karnovsky's fixative (2% paraformaldehyde and 1% glutaraldehyde in cacodylate buffer) for 30 min then osmicated in osmium tetroxide following standard conditions. The coverslips were embedded in LR-White and polymerized overnight at 4°C. Ultrathin sections were

cut with a glass knife and mounted on nickel grids. The grids were stained using the hanging method by suspension on drops of blocking buffer (10% goat serum in filtered PBS) for 1 hr, then primary antibody (7E11.C5 or IgG control) for up to 1 hr, rinsed by repeated hanging drop incubation in wash buffer, then incubated with a secondary anti-mouse Ig antibody labeled with a 10 nm gold bead (Amersham Life Science, Arlington Heights, IL). Following several rinses, the grids were counterstained by placement at the bottom of a drop of filtered uranyl acetate for 15 min in the dark then rinsed three times by dipping 20 times for each wash in filtered sterile water. After the final rinse, the grids were placed at the bottom of a drop of lead citrate and stained for 15 sec then rinsed three times as described above. The grids were air dried on filter paper then analyzed on a JEOL transmission electron microscope.

Serum Starvation Stimulation

LNCaP cells were grown to approximately 50% confluency on chambered slides as described above. The cells were washed three times with PBS warmed to 37°C, serum-free RPMI 1640 medium was added to each well, and the slides were incubated for an additional 48 hr at 37°C. Following serum starvation, the serum-free medium was removed by aspiration and replaced with RPMI 1640 medium supplemented with 5% calf serum and incubated at 37°C. Slides were removed at time points (0 min, 15 min, 30 min, 60 min, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 24 hr, and 48 hr) following serum starvation and fixed for 20 min in 10% buffered formalin and stored in PBS at 4°C. After all of the time points were collected and fixed, the cells were permeabilized briefly in 0.1% Triton-X100 in PBS for 5 min, then washed twice with PBS. Immunofluorescence staining was carried out as described above using MAb 7E11-C5.3 and an IgG1 isotype-matched control antibody. All assays were run in triplicate.

Mitochondrial Purification

Mitochondria were purified from LNCaP cells following a previously reported procedure [14]. Six 162 cm² tissue culture flasks were seeded with LNCaP cells and grown to confluency. The cells were harvested then pelleted by centrifugation. The cell pellet was resuspended in 6 ml 1 \times mitochondrial isolation buffer (MIB; 0.25 M sucrose, 40 mM Tris, pH 7.0, 0.1 mM EDTA) then dounce homogenized on ice with 40 up and down strokes. The homogenized material was centrifuged in a Beckman JA20 rotor at 2,000 g for 30

min at 4°C, and the supernatant was collected and centrifuged at 8,500 g for 35 min at 4°C. The supernatant from this step was discarded, and the pellet resuspended in 6 ml 1 × MIB and centrifuged at 8,500 g for 35 min. The supernatant from this step was discarded and the pellet resuspended in 6 ml 1 × MIB and dounce homogenized. This suspension represented the crude mitochondrial fraction. The crude mitochondrial fraction was applied to a two-step sucrose gradient composed of 10 ml of 25% sucrose in TE buffer (10 mM Tris, 0.1 mM EDTA) and 13 ml of 42.5% sucrose in TE buffer and centrifuged at 26,000 g for 75 min at 4°C. The percentage of sucrose in each solution was confirmed by a refractive index of 1.3775 and 1.4035, respectively. The mitochondria were collected at the interface of the two sucrose layers and diluted in two volumes TE buffer and applied to a second sucrose gradient purification. The mitochondria were again collected at the interface of the two sucrose layers, diluted in 2 volumes TE buffer then centrifuged at 22,000 g for 20 min at 4°C. The pellet from this step, representing the purified mitochondria, was resuspended in 2 volumes PBS and stored at -20°C until needed.

RESULTS

CAP Cellular Subfractionation

Following subcellular subfractionation procedures carried out on cultured LNCaP cells, the PSMA glycoprotein was detected by Western blot analysis in both the HM and the LM fractions at approximately 100 kDa (Fig. 1). The HM fraction also contained a higher molecular weight band which was barely detectable in the LM fraction, indicating that two pools of this molecule exist in LNCaP cells. A small amount of reactivity was also seen in the nuclear fraction but may be due to contamination. A panel of control antibodies, including antibodies against the PCNA, HSP60 (MU213), the transferrin receptor (OKT-9), and prostate-specific antigen (PSA), was utilized to monitor the efficiency of the fractionation (data not shown).

Immunofluorescence Microscopy of PSMA in LNCaP Cells

LNCaP cells grown on tissue culture slides were stained without fixation or permeabilization prior to immunofluorescence staining with MAb 7E11-C5.3 or a control antibody (OKT-9) specific for the extracellular domain of the transferrin receptor. The transferrin receptor antibody was able to bind its epitope on the extracellular face of the LNCaP cells, resulting in a ring of staining around the cells (Fig. 2A) as expected

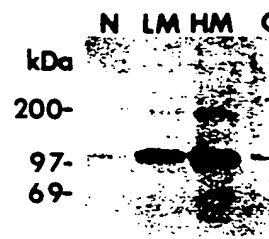


Fig. 1. Western blot analysis of cellular fractions. The majority of PSMA was found in the light membrane (LM) and heavy membrane (HM) fractions with very little in the nuclear (N) and cytoplasmic (C) fractions.

for this extracellularly expressed epitope. On the other hand, MAb 7E11-C5.3 was unable to bind to its epitope (Fig. 2B), indicating its epitope was intracellular.

To more definitively determine the localization of PSMA within the LNCaP cell line, cells grown on tissue culture slides were fixed with buffered formalin and permeabilized briefly in a weak detergent solution prior to staining. The isotype-matched control antibody did not stain the LNCaP cells (Fig. 2C) and all that was visible was the red counterstaining of the Evan's blue. Staining with PSA-5 showed a remarkable vesicular staining of the LNCaP cells (Fig. 2D), suggesting a localization in secretory vesicles. The transferrin receptor appeared to be predominantly localized to the plasma membrane (Fig. 2E), as demonstrated by the circumscription of the cell by the yellow fluorescence. The staining for PSMA with MAb 7E11-C5.3, however, appeared to be cytoplasmic and not localized at the plasma membrane or the nucleus (Fig. 2F). There was no circumscription of the cells as seen with the transferrin receptor and no vesicular staining similar to PSA.

A similar set of experiments were carried out using immunoperoxidase staining methods and visible light microscopy with identical results. The staining pattern for PSMA was observed to be cytoplasmic (Fig. 3A,B) while the transferrin receptor was restricted to the plasma membrane (Fig. 3C).

Immunoelectron Microscopy of PSMA in LNCaP Cells

A series of immunoelectron microscopy studies were then performed to definitively determine the

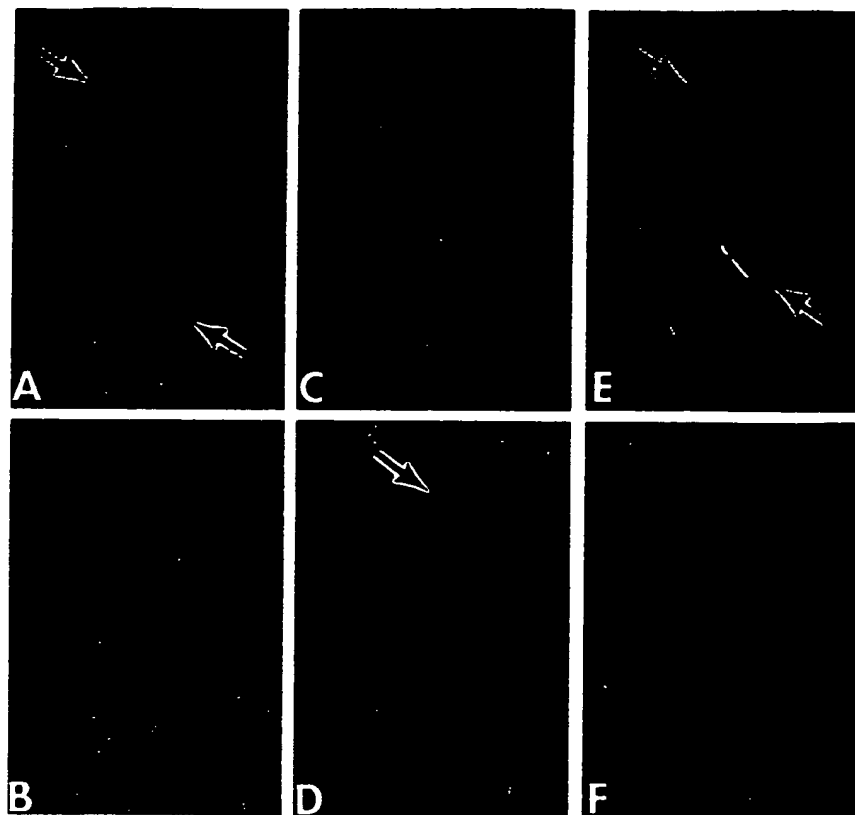


Fig. 2. Live and fixed cell immunofluorescent analysis of LNCaP cells. For the live cell assay, LNCaP cells were grown on tissue culture slides, washed, and reacted with MAb without fixation. **A:** Positive staining (yellow-green ring around cells indicated by arrows) of the extracellular domain of the transferrin receptor with MAb OKT-9. **B:** MAb 7E11-C5 did not react with LNCaP cells under the same conditions. Only the Evan's blue counterstain (red fluorescence) is observed. Fixed LNCaP cells were reacted

with either the IgG1 isotype-matched control (**C**), MAb PSA-5 (**D**), MAb OKT-9 (**E**), or MAb 7E11-C5 (**F**). The isotype-matched antibody control was negative while PSA-5 gave a very vesicular staining pattern (arrow). OKT-9 staining was similar to that observed in the live cell assay in that the staining was confined to the plasma membrane (arrows), whereas LNCaP cells stained with MAb 7E11-C5 appeared to be predominantly cytoplasmic and not localized to the nucleus or exclusively to the plasma membrane.

intracellular localization of PSMA within the cytoplasm of LNCaP cells. The first set of experiments utilized a chromagen (DAB) appearing as electron-dense particles to visualize PSMA staining. Figure 4 shows the positive staining of MAb 7E11-C5.3 at the cytoplasmic face of the cell membrane (CM) and microvilli (MV) (Fig. 4A,B) and within several mitochondria (Fig. 4G).

To more precisely demonstrate the localization of PSMA, a secondary MAb directly labeled with a 10 nm gold bead was used in place of chromagen staining. Immunogold staining with MAb 7E11-C5.3 also resulted in positive staining with a concentration of gold beads at the cytoplasmic face of the CM and near a gap junction (GJ) between two LNCaP cells (Fig. 4E)

and concentrated in and around mitochondria (Fig. 4H). When LNCaP cells were reacted with MAb OKT-9, immunogold staining was localized to the outer face of the plasma membrane (Fig. 4D). Specific staining of the plasma membrane or mitochondria was not observed when an isotype-matched MAb was used (Fig. 4C,F).

PSMA Identified in Mitochondria

Since two separate experiments suggested that PSMA was, in part, localized to the mitochondria of LNCaP cells, mitochondria were purified from whole cell lysates and analyzed for the presence of PSMA. The 100 kDa PSMA glycoprotein was present in the

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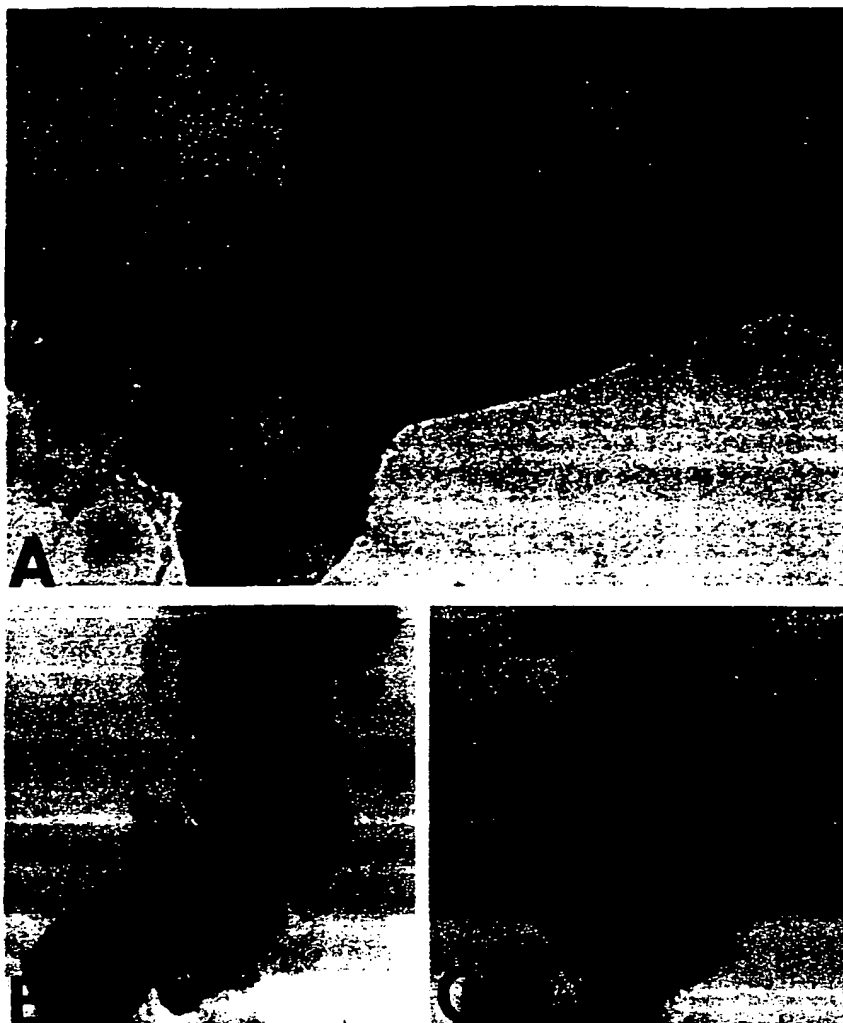


Fig. 3. Immunoperoxidase staining of LNCaP cells. A: Cultured LNCaP cells, fixed, and reacted with MAb 7E11-C5 show intense cytoplasmic staining. B: Cytospin preparation of fixed LNCaP cells reacted with MAb 7E11-C5 also shows intense cytoplasmic staining. C: Cytospin preparation of fixed LNCaP cells reacted with the

anti-transferrin receptor MAb OKT-9. In contrast to the cytoplasmic staining with MAb 7E11-C5, MAb OKT-9 results in the ring membrane staining pattern typical for the location of an extracellular membrane epitope.

LNCaP lysate (Fig. 5, lane 1), crude mitochondrial preparation (Fig. 5, lane 2), and purified mitochondria following several sucrose gradient centrifugation steps (Fig. 5, lane 3), adding further evidence that PSMA is associated with the mitochondria of LNCaP cells.

Further evidence for PSMA association with mitochondria was shown by copurification of a mitochondrial protein with PSMA. A third major band of an approximate molecular weight of 40 kDa was copuri-

fied with PSMA on a MAb 7E11-C5.3 affinity column when low detergent wash buffers were used on the column (Fig. 6, lane 1), but was not immunoreactive (lane 2). This band was blotted to nitrocellulose and subjected to amino acid sequence analysis. The amino acid sequence of this band was completely identical to the precursor form of the mitochondrial isoenzyme of aspartate aminotransferase (m-AST), which is also known as S glutamic oxalacetic transaminase (SGOT) (data not shown).

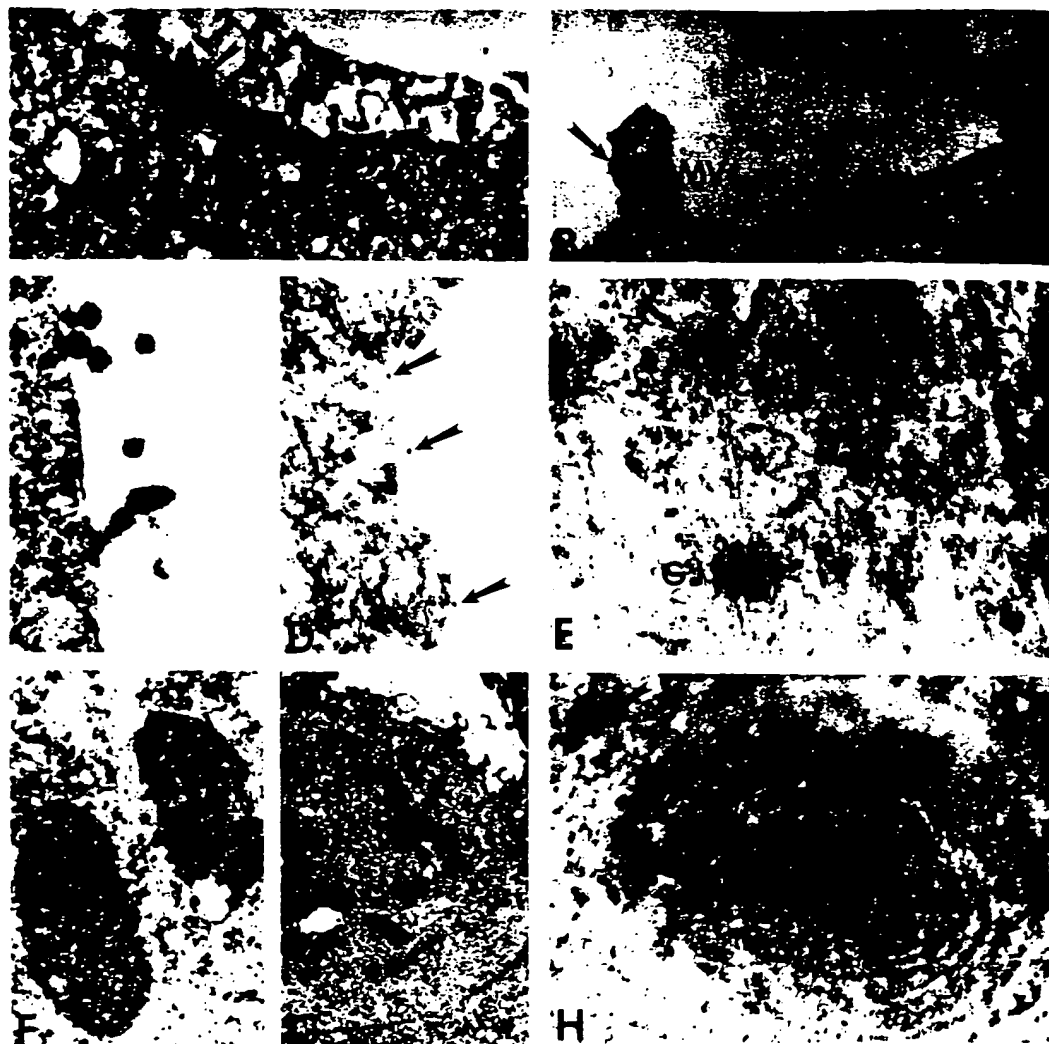


Fig. 4. Immunoelectron microscopy of LNCaP cells. Location of the PSMA-7E11-C5 complex was detected using either a peroxidase (DAB; Panels A, B, F, and G) or colloidal gold (Panels C-F) procedure as described in Materials and Methods. The PSMA-antibody complex was occasionally found at the cytoplasmic side of the cell membrane (CM) (A, arrow), and microvilli (MV; Panel B, arrow). No DAB or gold beads were found to be localized to the cell membrane in cells reacted with an isotype control MAb (negative control) (C). D: Location of gold beads at the outer face of the plasma membrane (arrows) of cells reacted with MAb OKT-9

(positive membrane control). The most common location of the PSMA-7E11-C5 complex was at the inner face of the plasma membrane (E) and in mitochondria (G, H). Panel E shows a cluster of gold beads under the cell membrane (CM) (arrow) near a gap junction (GJ). Panels F, G are examples of mitochondria reacted with either the isotype control MAb (F), or with MAb 7E11-C5 (G, H). Immune complexes were not detected with the control antibody (F), whereas PSMA was localized within and around (arrows, G and H) mitochondria with MAb 7E11-C5. Magnifications: $\times 13,000$ (G); $\times 28,000$ (C, F); $\times 81,000$ (B, D); $\times 97,000$ (A, E, H).

Effects of Serum Starvation and Stimulation on PSMA Localization

LNCaP cells examined after serum starvation followed by serum stimulation showed the typical cyto-

plasmic immunofluorescent staining pattern of PSMA observed under normal growth conditions, suggesting that the localization and expression of PSMA was not dependent on extracellular signals.

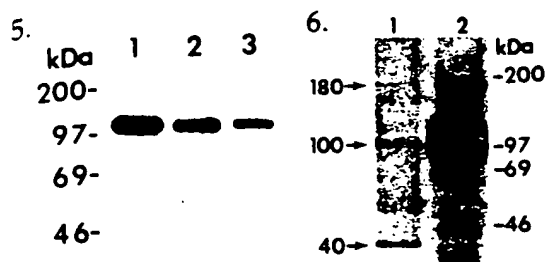


Fig. 5. Western blot analysis of crude and purified mitochondria. The mitochondria were purified by a series of centrifugation steps and sucrose gradient centrifugation. The LNCaP cell lysate contained a significant amount of the 100 kDa PSMA glycoprotein (lane 1) as did the crude mitochondrial preparation (lane 2) and the pure mitochondria following two sucrose gradient centrifugation purifications (lane 3), indicating that PSMA is found in the mitochondrial fraction of LNCaP cells.

Fig. 6. Identification of the mitochondrial subunit SGOT-AST copurified with PSMA. Affinity-purified PSMA was separated by gel electrophoresis and the gel either stained with coomassie blue (lane 1) or immunoblotted with MAb 7E11-C5 (lane 2). Both the 100 and 180 kDa PSMA bands were detected by staining and immunoblotting, with the 100 kDa band being highly reactive (lane 2). Note the prominent 40 kDa SGOT-AST band, which copurified with PSMA (lane 1), was not immunoreactive with MAb 7E11-C5 (lane 2).

DISCUSSION

PSMA is a transmembrane glycoprotein found to be predominately expressed by normal, benign, and malignant prostate epithelial cells and overexpressed in high-grade and metastatic prostate carcinomas [3]. The function of PSMA remains unknown. Recently, we reported that the antigenic epitope recognized by MAb 7E11-C5 was located on the intracellular or cytoplasmic domain of PSMA [7]. Interestingly, MAb 7E11-C5 conjugated to ^{111}In has been successfully used to image metastatic prostate carcinomas in vivo [4,5]. It is currently believed that for most antibodies to be effective for in vivo imaging of tumors, they must bind to an extracellular or surface membrane antigen. Since it appeared from our previous studies [3,7] that MAb 7E11-C5 bound to a cytoplasmic antigen, how does MAb 7E11-C5 image occult prostate carcinomas if it cannot penetrate the plasma membrane of the intact cancer cell? The present investigation was performed to address this issue and to provide definitive information about the cellular localization of PSMA. The LNCaP prostate cell line was selected for this study because it was the cell line used to generate MAb 7E11-C5.

Live cell immunofluorescence staining of LNCaP

cells showed that MAb 7E11-C5 was unable to bind its target epitope, in contrast to a MAb to the transferrin receptor which expectedly bound to its epitope at the extracellular face of the plasma membrane. When fixed and permeabilized LNCaP were stained with MAb 7E11-C5, a diffuse cytoplasmic staining pattern was observed. This cytoplasmic staining pattern was observed by both immunofluorescent and immunoperoxidase methods, suggesting that the cytoplasmic staining was not due to an artifact of the staining method. It may, nevertheless, be argued that the weak detergent permeabilization of the cells prior to staining caused the cell membrane to dissipate, resulting in the cytoplasmic staining pattern. However, cells treated in the same manner and stained with MAb OKT-9 to the transferrin receptor showed the typical membrane staining [15] around the circumference of the cells, indicating that detergent treatment was not sufficient to cause diffusion or loss of the membrane epitope. These findings strongly supported a cytoplasmic location of the PSMA epitope recognized by MAb 7E11-C5.

We next attempted to pinpoint the intracellular location of PSMA by performing cellular immunoelectron and subfractionation experiments. Immunoelectron microscopy of LNCaP cells showed that PSMA was in fact found at two locations inside the cell. The PSMA epitope recognized by MAb 7E11-C5.3 was repeatedly found localized at the cytoplasmic face of the plasma membrane, strongly supporting the conclusion of an intracellular epitope. The only distinct positive staining in addition to the plasma membrane was found within and surrounding the outer mitochondrial membrane. A localization solely at the cytoplasmic face of the cell membrane would result in a ringed staining identical to that of an extracellular epitope. However, the additional localization to mitochondria would explain the diffuse cytoplasmic appearance of the staining at the light microscopy level. Positive staining for PSMA at both the cell membrane and mitochondria was observed utilizing both indirect chromagen staining and direct staining with a gold-labeled secondary antibody. These identical results using multiple immunoelectron microscopy techniques, as well as the negative staining by an isotype-matched control antibody, suggest the staining was specific.

Although the controls used for the immunolocalization experiments strongly suggested that our conclusions for the intracellular location of PSMA were valid, we felt that further experiments were needed to prove that PSMA was found in or associated with the mitochondria. When purified mitochondria from LNCaP cells were tested by Western blot analysis, a high concentration of PSMA was observed. Although

the purity of the purified mitochondrial preparation was not examined by electron microscopy, the purification procedure used routinely yields a highly purified preparation [14]. Furthermore, it seems unlikely that a small amount of contamination with other cellular membranes would have given such a strong signal upon immunoblotting with MAb 7E11-C5.3.

The cellular subfractionation of LNCaP cells also clearly demonstrated two pools of PSMA; one pool in the LM fraction most likely consisting largely of plasma membrane components indicated by the high concentration of PSMA measured by ELISA and Western blot analyses; and the other in the HM fraction containing mostly intracellular organelles. Only very small amounts of PSMA were found in either the cytoplasmic or nuclear fractions. The HM fraction may also contain a significant number of plasma membrane proteins that happen to be contained in the rough ER as they transit from the ER to the membrane. The concentration of these plasma membrane components in the ER may be enhanced if there is a defect in trafficking of molecules through this pathway [16]. Alternatively, PSMA may be found in the mitochondria of LNCaP cells since the mitochondria make up a significant portion of the HM fraction as judged by the detection of significant concentrations of the mitochondrial-specific Hsp60 protein.

Collectively, the data from the different experiments described in this report strongly suggest the PSMA glycoprotein is localized at the plasma membrane with the epitope facing the cytoplasm and that it is also present within mitochondria. While it was quite unexpected to find a prostate-specific glycoprotein localized to an organelle considered to be rather generic in its constitution, there is precedent for the localization of plasma membrane glycoproteins to the mitochondria. For example, the Her2/neu oncogene product has also been shown to be localized at the plasma membrane and mitochondria [17]. Interestingly, like PSMA, a large percentage of Her2/neu staining in prostate and breast carcinoma cells appears to be cytoplasmic in nature [18,19] and not localized solely at the plasma membrane. This would seem to suggest there is a significant amount of communication between the extracellular environment and intracellular organelles. It is likely that a monitoring mechanism exists which may modify the metabolic activity of the organelle and that a significant number of plasma membrane receptors, including Her2/neu and PSMA, may be involved in this process. While such a mechanism may not have been proposed 10 years ago, a similar communication has been described among the mitochondria, peroxisomes, and the nucleus [20] and during starvation,

myocardial cells appear to have an adaptive interaction between the intracellular and extracellular environments [21]. Additionally, many studies have been carried out in recent years on the organization of the extracellular matrix, concluding that cells seem to be able to sense the macromolecular composition of the extracellular matrix and to modify their production of matrix components accordingly [22]. These studies indicate that a considerable amount of communication occurs between the different cellular components. Therefore, the suggestion that PSMA may be involved in subcellular communication is not without precedent.

Serum starvation or stimulation of LNCaP cells failed to alter the staining pattern of PSMA. It is possible that a signal at the cell membrane results in a sequestration of PSMA at the membrane and if the ligand for PSMA is not present in the tissue culture media, PSMA would remain spread throughout the cell. In fact, a detailed examination of MAb 7E11-C5.3 staining of prostate tissue showed some ductal epithelial cells to exhibit staining confined to the plasma membrane with no cytoplasmic staining [3]. This staining pattern may reflect the presence or absence of a possible PSMA ligand which has not been duplicated in the *in vitro* studies.

Although the results of this study do not provide a definitive answer to why ^{111}In -conjugated MAb 7E11-C5 (CYT-356) can image prostate carcinomas *in vivo*, it is doubtful CYT-356 is able to cross the plasma membrane since 7E11-C5.3 was not able to do so *in vitro* in living LNCaP cells. Due to the propensity of prostate cells to enter apoptosis [23], the most probable answer is that a significant number of cells within a tumor may be lysed following cell death, thereby uncovering the antigenic epitope. The number of apoptotic cells within a tumor may be substantially increased if the patient is on hormone ablation therapy since withdrawal of androgen hormones induces apoptosis [24], which may lead to an increase in the number of epitopes available to be recognized by CYT-356. Preliminary evidence suggests that hormone ablation therapy can upregulate the expression of PSMA [25] [Wright et al., unpublished observations], thereby supporting this possibility. Alternatively, as a tumor grows, a significant portion of the tumor volume may become necrotic [26], again allowing for access to the intracellular epitope. Both of these mechanisms would result in CYT-356 "seeing" only a small percentage of the cells within a given tumor mass resulting in a significantly low sensitivity. As a result, specific second-generation antibodies generated against the extracellular domain of PSMA may dramatically increase the sensitivity of imaging assays by increasing the number of cells which can be

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recognized by the antibody. This increased sensitivity may result in the detection of smaller metastatic foci containing a large percentage of intact cells which would not be discovered by MAb 7E11-C5. It must be stated in any discussion of PSMA second-generation antibodies, however, that the MAb 7E11-C5.3 may be the most selective antibody for prostate tissue. It is highly likely that many of the MABs generated against the extracellular domain of PSMA may be crossreactive with other cellular antigens. Therefore, it may not be possible to greatly improve the sensitivity of PSMA imaging over that which is obtained using 7E11-C5.3.

In summary, the results of this study strongly support the intracellular location of the PSMA epitope recognized by MAb 7E11-C5, not only to the cytoplasmic face of the plasma membrane but in association with mitochondria. This latter observation may offer a clue to the function of the PSMA glycoprotein. Further, since MAb 7E11-C5 cannot enter living LNCaP cells to reach its target epitope, it apparently binds to PSMA when it is released from cells undergoing apoptosis or necrosis. These observations would suggest that generating MABs to extracellular epitopes may enhance the use of antibody-directed imaging and therapy of prostate carcinomas, and possibly for development of other potential clinical applications of this novel prostate cancer biomarker.

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REFERENCES

1. Wingo PA, Tong T, Bolden S: Cancer statistics. *CA* 45: 8-30, 1995.
2. Horoszewicz JS, Kawinski E, Murphy GP: Monoclonal antibodies to a new antigenic marker in epithelial prostate cells and serum of prostatic cancer patients. *Anticancer Res* 7:927-936, 1987.
3. Wright GL Jr, Haley C, Beckett ML, Schellhammer PF: Expression of prostate specific membrane antigen (PSMA) in normal, benign, and malignant prostate tissues. *Urol Oncol* 1:18-28, 1995.
4. Kahn D, Williams RD, Seldin DW, Libertino JA, Hirschhorn M, Dreicer R, Weiner GJ, Bushnell D, Gulfo J: Radioimmunoscinigraphy with ¹¹¹Indium labeled CYT-356 for the detection of occult prostate cancer recurrence. *J Urol* 152:1490-1495, 1994.
5. Babaian RJ, Sayer J, Podoloff DA, Steelhammer LC, Bhadkamkar VA, Gulfo JV: Radioimmunoscinigraphy of pelvic lymph nodes with ¹¹¹indium-labeled monoclonal antibody CYT-356. *J Urol* 152:1952-1955, 1994.
6. Axelrod HRR, Gilman SC, D'Aleo CJ, Petrylack D, Reuter V, Gulfo JV, Saad A, Cordon-Cardo C, Scher HRI: Preclinical results and human immunohistochemical studies with 90Y-CYT-356: A new prostate cancer therapeutic agent. *J Urol* 147:361A (Abstract 596), 1992.
7. Troyer JK, Feng Q, Beckett ML, Wright GL Jr: Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate specific membrane antigen. *Urol Oncol* 1:29-37, 1995.
8. Shaulsky G, Ben-Ze'ev A, Rotter V: Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. *Oncogene* 5:1707-1711, 1990.
9. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC: Investigation of the subcellular distribution of the bcl-2 oncoprotein: Residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 53:4701-4714, 1993.
10. Akao Y, Otsuki Y, Kataoka S, Ito Y, Tsujimoto Y: Multiple subcellular localization of bcl-2: Detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. *Cancer Res* 54: 2468-2471, 1994.
11. Danscher G, Norgaard JOR: Light microscopic visualization of colloidal gold on resin-embedded tissue. *J Histochem Cytochem* 31:1394-1398, 1983.
12. Simsin JAV, Fenters R, Chao J: Electron microscopic immunostaining of kallikrein in rat submandibular glands. *J Histochem Cytochem* 31:301-306, 1983.
13. Song GX, Lin CT, Wu JY, Lam KW, Li CY, Yam LT: Immunoelectron microscopic demonstration of prostatic acid phosphatase in human hyperplastic prostate. *Prostate* 7:63-71, 1985.
14. Lazarus GM, Henrich JP, Kelly WG, Schmitz SA, Castora FJ: Purification and characterization of a type I DNA topoisomerase from calf thymus mitochondria. *Biochemistry* 26:6196-6203, 1987.
15. Karlsson GB, Platt FM: Analysis and isolation of human transferrin receptor using the OKT-9 monoclonal antibody covalently crosslinked to magnetic beads. *Anal Biochem* 199:219-222, 1991.
16. Hicke L, Schekman R: Molecular machinery required for protein transport from the endoplasmic reticulum to the Golgi complex. *Bioassays* 12:253-258, 1990.
17. DePotter CR, Quatacker J, Maertens G, Van Daele S, Pauwels C, Verhofstede C, Eechaute W, Roels H: The subcellular localization of the neu protein in human normal and neoplastic cells. *Int J Cancer* 44:969-974, 1989.
18. Wright GL Jr, Grob M, Huang CL, Troyer JK, Adam BL, Cazares L, Schellhammer PF: Comparison of HER2-neu in prostate and breast carcinomas. *Proc Am Assoc Cancer Res* 36:644 (Abstract 3837), 1995.
19. Wright GL Jr, Grob M, Troyer JK, Adam BL, Cazares L, Schellhammer PF: HER2-neu expression in prostate carcinomas is not the same as that found in breast carcinomas. *J Urol* 153:271A (Abstract 170), 1995.
20. Liao X, Butow RA: RTG1 and RTG2: Two yeast genes required for a novel path of communication from mitochondria to the nucleus. *Cell* 72:61-71, 1993.
21. Vandewoude MF, Buysens N: Effect of aging and malnutrition on rat myocardium. I. The myocyte. *Virchows Arch [A]* 421:179-188, 1992.



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<p>(21) International Application Number: PCT/EP92/03016</p> <p>(22) International Filing Date: 30 December 1992 (30.12.92)</p> <p>(30) Priority data: MI91A03513 31 December 1991 (31.12.91) IT</p> <p>(71) Applicant (for all designated States except US): BIOCINE SCLAVO SPA [IT/IT]; Via Fiorentina, 1, I-53100 Siena (IT).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): DOMENIGHINI, Mario [IT/IT]; Via Ungaretti, 17, I-53010 Quercegrossa (IT). RAPPUOLI, Rino [IT/IT]; Via Calamandrei, 39, I-53010 Quercegrossa (IT). PIZZA, Mariagrazia [IT/IT]; Via Colombini, 30, I-43100 Siena (IT). HOL, Wim [NL/ US]; 18332 57th Avenue, N.E., Seattle, WA 98155 (US).</p>		<p>(74) Agent: HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).</p> <p>(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>
<p>(54) Title: IMMUNOGENIC DETOXIFIED MUTANTS OF CHOLERA TOXIN AND OF THE TOXIN LT, THEIR PREPARATION AND THEIR USE FOR THE PREPARATION OF VACCINES</p> <p>(57) Abstract</p> <p>An immunogenic detoxified protein comprising the amino acid sequence of subunit A of cholera toxin (CT-A) or subunit A of an <i>Escherichia coli</i> heat labile toxin (LT-A) or a fragment thereof wherein one or more amino acids at, or in positions corresponding to Val-53, Ser-63, Val-97, Tyr-104 or Pro-106 are replaced with another amino acid or deleted. Examples of specific replacements include Val-53-Asp, Val-53-Glu, Val-53-Tyr, Ser-63-Lys, Val-97-Lys, Val-97-Tyr, Tyr-104-Lys, Tyr-104-Asp, Tyr-104-Ser, Pro-106-Ser. The immunogenic detoxified protein is useful as vaccine for <i>Vibrio cholerae</i> or an enterotoxigenic strain of <i>Escherichia coli</i> and is produced by recombinant DNA means by site-directed mutagenesis.</p> <div data-bbox="698 1134 1429 1890"> </div>		

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Immunogenic detoxified mutants of cholera toxin and of the toxin LT, their preparation and their use for the preparation of vaccines

5 Field of the Invention

The present invention relates to immunogenic detoxified proteins of cholera toxins (CT), or of heat labile toxins (LT) produced by the enterotoxigenic strains of *Escherichia coli* (*E.coli*) having substitutions at one or more of amino acids Val-53, Ser-63, Val-97, Tyr-104 or Pro-106 and to their use in vaccines which are useful for the prevention or treatment of cholera or enterotoxigenic *E.Coli* infections. The proteins can be suitably produced using recombinant DNA techniques by site-directed mutagenesis of DNA encoding the wild type toxins.

Background to the Invention

20 Cholera is a contagious disease widely distributed in the world, in particular in the Third World, where, in certain areas, it is endemic. The serious disorders which develop in the intestinal system prove fatal in a high percentage of the recorded cases of the disease.

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The etiological agent of cholera is the Gram-negative microorganism *Vibrio cholerae* (*V.cholerae*). This colonises the intestinal tract of individuals who have come into contact with it through ingestion of contaminated food or water, and multiplies to very high concentrations. The principal symptom is severe diarrhoea as a result of which the patient can lose as much as 10-15 litres of liquids per day via the faeces. As a result of the severe dehydration and loss of electrolytes, the patient does not withstand the infection in 50-60% of cases, and dies. The diarrhoea caused by *V.cholerae* is due to the secretion of cholera toxin, CT, which acts by stimulating the activity of the adenylate cyclase enzyme so as to induce disturbances at cell level.

Although cholera can be effectively cured by controlled and intense rehydration, the distribution of a vaccine is desirable with a view to complete control and future eradication of the disease.

5

At the present time, there exists a vaccination against cholera, consisting of parenteral administration of killed bacteria. Although some countries insist on vaccination against the disease, there are serious doubts as to its real usefulness, given that the current cellular vaccine protects
10 against the consequences of the infection in only 50% of the cases and that the protection is also extremely limited in duration, to less than 6 months.

15 In Bangladesh, an experimental trial is in progress (1990-92) of an oral vaccine consisting of killed bacteria with the addition of subunit B of cholera toxin, which is known to be highly immunogenic. This product succeeds in inducing lasting protection, without special side effects (Holmgren
20 J., Clemens J., Sack DA., Sanchez J. and Svennerholm AM; "Oral Immunization against cholera" Curr. Top. Microbiol. Immunol. (1988), 146, 197-204).

Cholera toxin resembles the heat labile toxins of
25 enterotoxigenic strains of *Escherichia coli* in amino acid sequence, structure and mode of action.

The consequences of infection with an enterotoxigenic strain of *E.coli* are similar to, though less serious than, those of
30 cholera, and consist of severe diarrhoea and intestinal disorders.

The CT and LT toxins all comprise a single A subunit (or protomer A) responsible for the enzymic activity of the
35 toxin (herein CT-A or LT-A) and five identical B subunits (or protomer B) which are involved in the binding of the toxin to the intestinal epithelial cells (herein CT-B or LT-B).

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The A subunit penetrates the cell membrane and causes activation of adenylate cyclase by NAD-dependent ADP-ribosylation of a GTP-binding protein which controls the activity of the enzyme. The clinical effect of this is to cause massive fluid loss into the intestine.

Considerable research has been conducted on cholera toxin and the *E. coli* heat labile toxins.

10

The sequence of CT is known and has been described (Mekalanos J.J. et al Nature 306, page 551 (1983)).

The sequence of LT from enterotoxigenic strains of *E. coli* is, as mentioned, 80% homologous to CT and it too is known and described in the scientific literature. Spicer E.K. et al (Biol. Chem. 257 p. 5716-5721 (1982)) describe the amino acid sequence of the A sub unit of the heat labile toxin from an enterotoxigenic strain of *E. coli* found in pigs.

20

A bacterial chromosomal form of LT has been identified and sequenced by Pickett C.L. et al (J. Bacteriol. 169, 5180-5187, (1987)).

25 The sequence of the A subunit of LT from a strain of *E. coli* known to affect humans has also been sequenced (Yamamoto et al, J. Biol. Chem., 259, 5037-5044, (1984)).

In view of the potential clinical significance of a vaccine against cholera and enterotoxigenic bacteria there is a continuing and great interest in producing a detoxified toxin capable of immunising against cholera and enterotoxigenic bacteria. The techniques of genetic engineering allow specific mutations to be introduced into the genes encoding the toxins and the production of the mutated toxins using now conventional techniques of gene expression and protein purification.

Various groups have attempted to identify mutations of the genes, which involve loss of the toxicity characteristics of the encoded proteins. The studies are predominantly being carried out in respect of the gene for the toxin LT, from *E. coli*.

Harford, S. et al (Eur. J. Biochem. 183, page 311 (1989)) describe the production of a toxoid by *in vitro* mutagenesis of the LT-A gene from *E. coli* pathogenic for pigs. The resulting successful mutation contained a Ser-61-Phe substitution and a Gly-79-Lys substitution, the former being considered the more important. Harford et al suggest that, because of the similarities between the LT-A genes in *E. coli* pathogenic to humans and pigs and the CT-A gene, and because the toxins are thought to operate by a common mechanism, it may be possible to produce a cholera holotoxoid by introducing the Ser-61-Phe mutation into the CT-A gene.

Tsuji, T. et al (J. Biol. Chem. 265, p. 22520 (1990)) describe the mutation of the LT-A gene from plasmid EWD299 to produce a single substitution Glu-112-Lys which affects the toxicity of the mutant LT yet does not change the immunogenicity of the protein.

Grant, C.C.R. et al (Abstract B289 of the 92nd General Meeting of the American Society for Microbiology, 26-30th May 1992) describe conservative substitutions of histidines at 44 and 70 and tryptophan at 127 in LT-A which result in significant reductions in enzymic activity.

Some work has been conducted on mutations to CT.

Kaslow, H.R. et al (Abstract B291 of the 92nd General Meeting of the American Society for Microbiology, 26-30th May 1992) describe mutating Asp-9 and His-44 and truncating after amino acid 180 in CT-A which all essentially eliminate activity. Mutating Arg-9 is said to markedly attenuate activity. Mutating other amino acid sites had little effect

on toxicity.

Burnette, W.N. et al (Inf. and Immun. 59(11), 4266-4270, (1991)) describe site-specific mutagenesis of CT-A to
5 produce an Arg-7-Lys mutation paralleling that of a known detoxifying mutation in the A subunit of the *Bordetella pertussis* toxin. The mutation resulted in the complete abolition of detectable ADP-ribosyltransferase activity.

10 International patent application WO 92/19265 (Burnette, Kaslow and Amgen Inc.) describes mutations of CT-A at Arg-7, Asp-9, Arg-11, His-44, His-70 and Glu-112.

Mutations at Glu-110 (LT and CT) and Arg-146 (LT) have also
15 been described in the literature (Lobet, Inf. Immun., 2870, 1991; Lai, Biochem. Biophys. Res. Comm. 341 1983; Okamoto J. Bacteriol. 2208, 1988).

The crystal structure of LT has been determined by Sixma et
20 al (Nature, 351, 371-377, May 1991) and confirms the mutagenesis results described earlier in the literature, explaining structurally the significance of Glu-112 and Ser-61 in activity of the A sub unit and suggesting that His-44, Ser-114 and Arg-54 which are in the immediate
25 neighbourhood may be important for catalysis or recognition.

Summary of the invention

It has now been discovered by further and more detailed
30 analysis of the structure of the toxins that certain further amino acids in the sequences of CT-A and LT-A are in positions capable of decreasing the enzymatic activity of CT and LT when mutated suitably, individually or in conjunction with other mutations.

35

The object of the present invention is to provide a vaccine which gives total protection against cholera or enterotoxigenic *E. coli*, by means of a second generation

product consisting of a single antigen, a toxoid derived from CT or LT, which has been detoxified genetically.

The genetic detoxification of CT or LT retains the immunogenic properties of the toxoid whilst providing a significantly reduced and preferably absent toxicity.

According to a first aspect of the invention there is provided an immunogenic detoxified protein comprising the amino acid sequence of subunit A of a cholera toxin (CT-A) or a fragment thereof or subunit A of an *Escherichia coli* heat labile toxin (LT-A) or a fragment thereof, wherein one or more amino acids at, or in positions corresponding to Val-53, Ser-63, Val-97, Tyr-104 or Pro-106 are replaced with another amino acid.

The replaced amino acids are at locations in the sequences of CT-A or an LT-A which are conserved both in the amino acid sequence and structurally and are thus common to CT and the various LTs.

The immunogenic detoxified protein of the invention adopts substantially the same structural conformation as the wild type naturally occurring toxins. It is immunologically active and cross reacts with antibodies to the wild type toxins.

In this specification, references to CT and LT encompass the various naturally occurring strain variants as well other variants encompassing changes from the sequences disclosed herein which do not affect the immunogenicity of the assembled toxoid.

In this specification, references to amino acid coordinates such as "Val-97" connote the amino acid at that position in the sequence of the mature cholera toxin subunit A (CT-A), that is without the signal sequence (see Figure 1).

Where the specification refers to an LT-A, the amino acid coordinates refer to the corresponding position in CT-A as shown in Figure 1.

5 Thus, for example, Val-53 in CT corresponds to Val-52 in the LT1 subunit and Ser-63 in CT corresponds to Ser-62 in LT1, there being a single amino acid difference in numbering up to amino acid 89 of the LT1 sequence. Val-97 in the CT sequence corresponds to Val-93 in the LT1 sequence because
10 of the four amino acid difference at that point in the sequence.

In addition, the immunogenic detoxified protein of the invention may include other mutations such as, for example,
15 substitutions at one or more of Arg-7, Asp-9, Arg-11, His-44, Arg-54, Ser-61, His-70, His-107, Glu-110, Glu-112, Ser-114, Trp-127, Arg-146 or Arg-192.

The amino acid substituted for the wild type amino acid may
20 be a naturally occurring amino acid or may a modified or synthetic amino acid. The substitution may involve deletion of an amino acid altogether provided that the mutant retains the necessary immunogenic properties and exhibits a substantially reduced toxicity.

25 Substitutions which alter the amphotericity and hydrophilicity whilst retaining the steric effect of the substituting amino acid as far as possible are generally preferred.

30 Preferred substitutions include: Val-53-Asp, Val-53-Glu, Val-53-Tyr, Ser-63-Lys, Val-97-Lys, Val-97-Tyr, His-107-Glu, Tyr-104-Lys, Tyr-104-Asp, Tyr-104-Ser, Pro-106-Ser, Ser-114-Glu, Ser-114-Lys.

35 As used herein, the term "detoxified" means that the immunogenic composition exhibits a substantially lower toxicity relative to its naturally occurring toxin

counterpart. The substantially lower toxicity should be sufficiently low for the protein to be used in an immunogenic composition in an immunologically effective amount as a vaccine with causing significant side effects.

5 For example, the immunogenic detoxified protein should have a toxicity of less than 0.01% of the naturally occurring toxin counterpart. The toxicity may be measured in mouse CHO cells or preferably by evaluation of the morphological changes induced in Y1 cells. The term "toxoid" means a

10 genetically detoxified toxin.

The immunogenic protein may be a CT or LT subunit A toxoid, but is preferably an assembled toxin molecule comprising a mutated CT-A or LT-A subunit and five B subunits of CT or

15 LT. The B subunit may be a naturally occurring subunit or may itself be mutated.

The immunogenic protein is preferably a naturally occurring CT-A or an LT-A suitably modified as described above.

20 However, conservative amino acid changes may be made which do not affect the immunogenicity or the toxicity of immunogenic protein and preferably do not affect the ability of the immunogenic protein to form complete toxin with B subunit protein. Also, the immunogenic protein may be a

25 fragment of CT-A or an LT-A provided that the fragment is immunogenic and non toxic and contains at least one of the conserved regions containing one of the mutations according to the invention.

30 According to a second aspect of the invention, there is provided an immunogenic composition for use as a vaccine comprising an immunogenic detoxified protein of the first aspect of the invention and a pharmaceutically acceptable carrier.

35

The immunogenic composition may additionally contain one or more adjuvants and/or pharmaceutically acceptable diluents.

The invention also provides a vaccine composition comprising an immunogenic detoxified protein according to the first aspect of the invention and a pharmaceutically acceptable carrier. The vaccine composition may further comprise an
5 adjuvant.

According to a third aspect of the invention, there is provided a method of vaccinating a mammal against *Vibrio cholerae* or an enterotoxigenic strain of *Escherichia coli*
10 comprising administering an immunologically effective amount of an immunogenic detoxified protein according to the first aspect of the invention.

The immunogenic detoxified proteins of the invention may be
15 synthesised chemically using conventional peptide synthesis techniques, but are preferably produced by recombinant DNA means.

According to a fourth aspect of the invention there is
20 provided a DNA sequence encoding an immunogenic detoxified protein according to the first aspect of the invention.

Preferably the DNA sequence contains a DNA sequence encoding a complete CT or LT comprising DNA encoding both the
25 detoxified subunit A and subunit B in a polycistronic unit. Alternatively, the DNA may encode only the detoxified subunit A.

According to a fifth aspect of the invention, there is
30 provided a vector carrying a DNA according to the fourth aspect of the invention.

According to a sixth aspect of the invention, there is provided a host cell line transformed with the vector
35 according to the fifth aspect of the invention.

The host cell may be any host capable of producing CT or LT but is preferably a bacterium, most suitably *E.coli* or

V.cholerae suitable engineered to produce the desired immunogenic detoxified protein.

In a further embodiment of the sixth aspect of the invention, the host cell may itself provide a protective species, for example an *E.coli* or *V.cholerae* strain mutated to a phenotype lacking wild type LT or CT and carrying and expressing an immunogenic detoxified protein of the first aspect of the invention.

10

In a further embodiment of the sixth aspect of the invention the host cell is capable of expressing a chromosomal LT-A gene according to the first aspect of the invention.

15 According to a seventh aspect of the invention, there is provided a process for the production of an immunogenic detoxified protein according to the first aspect of the invention comprising culturing a host cell according to the sixth aspect of the invention.

20

According to a eighth aspect of the invention there is provided a process for the production of DNA according to the fourth aspect of the invention comprising the steps of subjecting a DNA encoding a CT-A or an LT-A or a fragment thereof to site-directed mutagenesis.

25

According to a ninth aspect of the invention there is provided a process for the formulation of a vaccine comprising bringing an immunogenic detoxified protein according to the first aspect of the invention into association with a pharmaceutically acceptable carrier and optionally with an adjuvant.

30

Industrial Applicability

35

The immunogenic detoxified protein of the invention constitutes the active component of a vaccine composition useful for the prevention and treatment of cholera

infections or infections by enterotoxigenic strains of *E.coli*. The compositions are thus applicable for use in the pharmaceutical industry.

5 Brief Description of the Drawings

Figure 1 shows the amino acid sequences of the wild type subunit A from:

- 10 i) cholera toxin (CT - Mekalanos et al op cit),
- ii) heat labile toxin from an *E.coli* strain found in man (LT1_1 - Yamamoto et al op cit)
- iii) heat labile toxin from an *E.coli* strain found in pigs (LT1- Spicer et al op cit), and
- 15 iv) heat labile toxin from a chromosomal source (LT1_1 - Pickett et al op cit)

The signal sequences are not shown.

- 20 In Figure 1, the conventional single letter amino acid code is used. The symbol "." denotes an absent amino acid and acts as a typographical spacer to ensure that the sequences remain in alignment for ease of comparison. The symbol "-"
- 25 " indicates an amino acid in the sequences of LT1 and LT2 which is identical to the corresponding amino acid in CT. The numbers against each line are the amino acid number of the first amino acid on that line.

- 30 In Figure 1 the positions of the mutations of the present invention are shown underlined.

Figures 2a and 2b are comparisons of the amino acid and DNA sequences of the A sub units of LT1 and CT.

- 35 Figure 3 is a restriction map of plasmid EWD299 (Dallas et al), bearing the LT-A gene.

Detailed Description of Embodiments of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989); DNA CLONING, VOLUMES I AND II (D.N Glover ed. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), Mayer and Walker, eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir and C. C. Blackwell eds 1986).

Standard abbreviations for nucleotides and amino acids are used in this specification. All publications, patents, and patent applications cited herein are incorporated by reference.

In particular, the following amino acid abbreviations are used:

Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp

	Cysteine	C	Cys
	Glycine	G	Gly
	Glutamic Acid	E	Glu
	Glutamine	Q	Gln
5	Histidine	H	His
	Isoleucine	I	Ile
	Leucine	L	Leu
	Lysine	K	Lys
	Methionine	M	Met
10	Phenylalanine	F	Phe
	Proline	P	Pro
	Serine	S	Ser
	Threonine	T	Thr
	Tryptophan	W	Trp
15	Tyrosine	Y	Tyr
	Valine	V	Val

As mentioned above examples of the immunogenic detoxified protein that can be used in the present invention include
20 polypeptides with minor amino acid variations from the natural amino acid sequence of the protein other than at the sites of mutation specifically mentioned.

A significant advantage of producing the immunogenic
25 detoxified protein by recombinant DNA techniques rather than by isolating and purifying a protein from natural sources is that equivalent quantities of the protein can be produced by using less starting material than would be required for isolating the protein from a natural source. Producing the
30 protein by recombinant techniques also permits the protein to be isolated in the absence of some molecules normally present in cells. Indeed, protein compositions entirely free of any trace of human protein contaminants can readily be produced because the only human protein produced by the
35 recombinant non-human host is the recombinant protein at issue. Potential viral agents from natural sources and viral components pathogenic to humans are also avoided. Also, genetically detoxified toxin are less likely to revert

to a toxic from than more traditional, chemically detoxified toxins.

Pharmaceutically acceptable carriers include any carrier
5 that does not itself induce the production of antibodies
harmful to the individual receiving the composition.
Suitable carriers are typically large, slowly metabolized
macromolecules such as proteins, polysaccharides, polylactic
acids, polyglycolic acids, polymeric amino acids, amino acid
10 copolymers, lipid aggregates (such as oil droplets or
liposomes) and inactive virus particles. Such carriers are
well known to those of ordinary skill in the art.
Additionally, these carriers may function as
immunostimulating agents (adjuvants).

15

Preferred adjuvants to enhance effectiveness of the compo-
sition include, but are not limited to: aluminum salts
(alum) such as aluminium hydroxide, aluminium phosphate,
aluminium sulfate etc., oil emulsion formulations, with or
20 without other specific immunostimulating agents such as
muramyl peptides or bacterial cell wall components, such as
for example (1) MF59 (Published International patent
application WO-A-90/14837, containing 5% Squalene, 0.5%
Tween® 80, 0.5% Span® 85 (optionally containing various
25 amounts of MTP-PE (see below), although not required)
formulated into submicron particles using a microfluidizer
such as Model 110Y microfluidizer (Microfluidics, Newton, MA
02164), (2) SAF, containing 10% squalene, 0.4% Tween 80, 5%
pluronic-blocked polymer L121, and thr-MDP (see below)
30 either microfluidized into a submicron emulsion or vortexed
to generate a larger particle size emulsion, and (3) RIBI™
adjuvant system (RAS) (Ribi Immunochem, Hamilton, MT)
containing 2% Squalene, 0.2% Tween® 80 and one or more
bacterial cell wall components from the group consisting of
35 monophosphoryl lipid A (MPL), trehalose dimycolate (TDM),
and cell wall skeleton (CWS) preferably MPL+CWS (Detox™),
muramyl peptides such as N-acetyl-muramyl-L-threonyl-D-
isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-iso-

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glutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE) etc., and cytokines, such as interleukins (IL-1, IL-2 etc) macrophage colony stimulating factor (M-CSF), tumour necrosis factor (TNF) etc. Additionally, saponin adjuvants, such as Stimulon[™] (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMS (immunostimulating complexes). Furthermore, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) may be used. Alum and MF59 are preferred.

The immunogenic compositions (e.g. the antigen, pharmaceutically acceptable carrier and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., nonhuman primate, primate, etc.), the capacity of the individual's immune

system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in
5 a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally, e.g. by injection either subcutaneously or
10 intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunc-
15 tion with other immunoregulatory agents.

The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or
20 manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

25 The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It
30 also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g.,
35 methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins

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(including for e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

A "replicon" is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc. that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control. This may include selectable markers.

A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control

sequences.

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is translated into a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, cDNA, and recombinant polynucleotide sequences.

"PCR" refers to the technique of polymerase chain reaction as described in Saiki, et al., Nature 324:163 (1986); and Scharf et al., Science (1986) 233:1076-1078; and U.S. 4,683,195; and U.S. 4,683,202.

As used herein, x is "heterologous" with respect to y if x is not naturally associated with y in the identical manner; i.e., x is not associated with y in nature or x is not associated with y in the same manner as is found in nature.

"Homology" refers to the degree of similarity between x and y. The correspondence between the sequence from one form to another can be determined by techniques known in the art. For example, they can be determined by a direct comparison of the sequence information of the polynucleotide. Alternatively, homology can be determined by hybridization of the polynucleotides under conditions which form stable duplexes between homologous regions (for example, those which would be used prior to S_1 digestion), followed by digestion with single-stranded specific nuclease(s), followed by size determination of the digested fragments.

As used herein, the term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term
5 also does not refer to or exclude post expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid
10 (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

15 A polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids,
20 and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.

25

The protein may be used for producing antibodies, either monoclonal or polyclonal, specific to the protein. The methods for producing these antibodies are known in the art.

30 "Recombinant host cells", "host cells," "cells," "cell cultures," and other such terms denote, for example, microorganisms, insect cells, and mammalian cells, that can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the
35 original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due

to natural, accidental, or deliberate mutation. Examples for mammalian host cells include Chinese hamster ovary (CHO) and monkey kidney (COS) cells.

5 Specifically, as used herein, "cell line," refers to a population of cells capable of continuous or prolonged growth and division in vitro. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes
10 can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants. The term "cell lines" also includes
15 immortalized cells. Preferably, cell lines include nonhybrid cell lines or hybridomas to only two cell types.

As used herein, the term "microorganism" includes prokaryotic and eukaryotic microbial species such as
20 bacteria and fungi, the latter including yeast and filamentous fungi.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective
25 of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

30 By "genomic" is meant a collection or library of DNA molecules which are derived from restriction fragments that have been cloned in vectors. This may include all or part of the genetic material of an organism.

35 By "cDNA" is meant a complementary DNA sequence that hybridizes to a complementary strand of DNA.

By "purified" and "isolated" is meant, when referring to a polypeptide or nucleotide sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. The term
5 "purified" as used herein preferably means at least 75% by weight, more preferably at least 85% by weight, more preferably still at least 95% by weight, and most preferably at least 98% by weight, of biological macromolecules of the same type present (but water, buffers, and other small
10 molecules, especially molecules having a molecular weight of less than 1000, can be present).

Once the appropriate coding sequence is isolated, it can be expressed in a variety of different expression systems; for
15 example those used with mammalian cells, baculoviruses, bacteria, and yeast.

i. Mammalian Systems

20 Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiating
25 region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian
30 promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation [Sambrook et al. (1989) "Expression of Cloned
35 Genes in Mammalian Cells." In Molecular Cloning: A Laboratory Manual, 2nd ed.].

Mammalian viral genes are often highly expressed and have a

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broad host range; therefore sequences encoding mammalian viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter (Ad 5 MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallothionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible), depending on the promoter can be 10 induced with glucocorticoid in hormone-responsive cells.

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA 15 sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or 20 flipped orientation, or at a distance of more than 1000 nucleotides from the promoter [Maniatis et al. (1987) Science 236:1237; Alberts et al. (1989) Molecular Biology of the Cell, 2nd ed.]. Enhancer elements derived from viruses may be particularly useful, because they usually have a 25 broader host range. Examples include the SV40 early gene enhancer [Dijkema et al (1985) EMBO J. 4:761] and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus [Gorman et al. (1982b) Proc. Natl. Acad. Sci. 79:6777] and from human cytomegalovirus 30 [Boshart et al. (1985) Cell 41:521]. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion [Sassone-Corsi and Borelli (1986) Trends Genet. 2:215; Maniatis et al. (1987) Science 236:1237].

35

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-

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terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide.

5

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either in vivo or in vitro. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

20 Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation [Birnstiel et al. (1985) Cell 41:349; Proudfoot and Whitelaw (1988) "Termination and 3' end processing of eukaryotic RNA. In Transcription and splicing (ed. B.D. Hames and D.M. Glover); Proudfoot (1989) Trends Biochem. Sci. 14:105]. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40 [Sambrook et al (1989) "Expression of cloned genes in cultured mammalian cells." In Molecular Cloning: A Laboratory Manual].

Some genes may be expressed more efficiently when introns

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(also called intervening sequences) are present. Several cDNAs, however, have been efficiently expressed from vectors that lack splicing signals (also called splice donor and acceptor sites) [see e.g., Gething and Sambrook (1981) Nature 293:620]. Introns are intervening noncoding sequences within a coding sequence that contain splice donor and acceptor sites. They are removed by a process called "splicing," following polyadenylation of the primary transcript [Nevins (1983) Annu. Rev. Biochem. 52:441; Green (1986) Annu. Rev. Genet. 20:671; Padgett et al. (1986) Annu. Rev. Biochem. 55:1119; Krainer and Maniatis (1988) "RNA splicing." In Transcription and splicing (ed. B.D. Hames and D.M. Glover)].

15 Usually, the above described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be

20 included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from

25 animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 [Gluzman (1981) Cell 23:175] or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen.

30 Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a procaryotic host for cloning

35 and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 [Kaufman et al. (1989) Mol. Cell. Biol. 9:946 and pHEBO [Shimizu et al. (1986) Mol. Cell. Biol. 6:1074].

The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

10

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), and a number of other cell lines.

ii. Baculovirus Systems

20

The polynucleotide encoding the protein can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques which are known in the art.

25

Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene in to the baculovirus genome); and appropriate insect host cells and growth media.

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild type viral

genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods
5 for baculovirus/insect cell expression systems are commercially available in kit form from, inter alia, Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, Texas Agricultural
10 Experiment Station Bulletin No. 1555 (1987) (hereinafter "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above described components,
15 comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are usually assembled into an intermediate transplacement construct (transfer vector). This construct may contain a single gene and operably linked regulatory elements;
20 multiple genes, each with its own set of operably linked regulatory elements; or multiple genes, regulated by the same set of regulatory elements. Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (e.g.,
25 plasmids) capable of stable maintenance in a host, such as a bacterium. The replicon will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

30 Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which
35 introduces a BamHI cloning site 32 basepairs downstream from the ATT; see Luckow and Summers, Virology (1989) 17:31.

The plasmid usually also contains the polyhedrin

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polyadenylation signal (Miller et al. (1988) Ann. Rev. Microbiol., 42:177) and a procaryotic ampicillin-resistance (amp) gene and origin of replication for selection and propagation in E. coli.

5

Baculovirus transfer vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable of binding a baculovirus RNA polymerase and initiating the downstream (5' to 3') transcription of a
10 coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription
15 initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

20 Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein, Friesen et al., (1986) "The Regulation of Baculovirus Gene Expression," in:
25 The Molecular Biology of Baculoviruses (ed. Walter Doerfler); EPO Publ. Nos. 127 839 and 155 476; and the gene encoding the p10 protein, Vlak et al., (1988), J. Gen. Virol. 69:765.

30 DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell et al. (1988) Gene, 73:409). Alternatively, since the signals for mammalian cell posttranslational modifications, (such as
35 signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the

invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human α -interferon, Maeda et al., (1985), Nature 315:592; human gastrin-releasing peptide, Lebacqz-Verheyden et al., (1988), Molec. Cell. Biol. 8:3129; human IL-2, Smith et al., (1985) Proc. Nat'l Acad. Sci. USA, 82:8404; mouse IL-3, (Miyajima et al., (1987) Gene 58:273; and human glucocerebrosidase, Martin et al. (1988) DNA, 7:99, can also be used to provide for secretion in insects.

10

A recombinant polypeptide or polyprotein may be expressed intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of nonfused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by in vitro incubation with cyanogen bromide.

20

Alternatively, recombinant polyproteins or proteins which are not naturally secreted can be secreted from the insect cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the translocation of the protein into the endoplasmic reticulum.

30

After insertion of the DNA sequence and/or the gene encoding the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild type baculovirus -- usually by co-transfection. The promoter and transcription termination sequence of the construct will usually comprise a 2-5kb section of the baculovirus genome.

35

Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summers and Smith supra; Ju et al. (1987); Smith et al., Mol. Cell. Biol. (1983) 3:2156; and Luckow and Summers
5 (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. Miller et al., (1989), Bioessays 4:91. The DNA sequence, when
10 cloned in place of the polyhedrin gene in the expression vector, is flanked both 5' and 3' by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter.

15 The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Homologous recombination occurs at low frequency (between about 1% and about 5%); thus, the majority of the virus produced after cotransfection is still
20 wild-type virus. Therefore, a method is necessary to identify recombinant viruses. An advantage of the expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein, which is produced by the native virus, is produced at very high
25 levels in the nuclei of infected cells at late times after viral infection. Accumulated polyhedrin protein forms occlusion bodies that also contain embedded particles. These occlusion bodies, up to 15 μ m in size, are highly refractile, giving them a bright shiny appearance that is
30 readily visualized under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells by techniques known to those skilled in the
35 art. Namely, the plaques are screened under the light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies. "Current Protocols in Microbiology" Vol. 2 (Ausubel

et al. eds) at 16.8 (Supp. 10, 1990); Summers and Smith, supra; Miller et al. (1989).

Recombinant baculovirus expression vectors have been
5 developed for infection into several insect cells. For
example, recombinant baculoviruses have been developed for,
inter alia: Aedes aegypti, Autographa californica, Bombyx
mori, Drosophila melanogaster, Spodoptera frugiperda, and
Trichoplusia ni (PCT Pub. No. WO 89/046699; Carbonell et
10 al., (1985) J. Virol. 56:153; Wright (1986) Nature 321:718;
Smith et al., (1983) Mol. Cell. Biol. 3:2156; and see
generally, Fraser, et al. (1989) In Vitro Cell. Dev. Biol.
25:225).

15 Cells and cell culture media are commercially available for
both direct and fusion expression of heterologous
polypeptides in a baculovirus/expression system; cell
culture technology is generally known to those skilled in
the art. See, e.g., Summers and Smith supra.

20 The modified insect cells may then be grown in an
appropriate nutrient medium, which allows for stable
maintenance of the plasmid(s) present in the modified insect
host. Where the expression product gene is under inducible
25 control, the host may be grown to high density, and
expression induced. Alternatively, where expression is
constitutive, the product will be continuously expressed
into the medium and the nutrient medium must be continuously
circulated, while removing the product of interest and
30 augmenting depleted nutrients. The product may be purified
by such techniques as chromatography, e.g., HPLC, affinity
chromatography, ion exchange chromatography, etc.;
electrophoresis; density gradient centrifugation; solvent
extraction, or the like. As appropriate, the product may be
35 further purified, as required, so as to remove substantially
any insect proteins which are also secreted in the medium or
result from lysis of insect cells, so as to provide a
product which is at least substantially free of host debris,

e.g., proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host cells derived from the transformants are incubated under conditions which allow expression of the recombinant protein encoding sequence. These conditions will vary, dependent upon the host cell selected. However, the conditions are readily ascertainable to those of ordinary skill in the art, based upon what is known in the art.

10

iii. Bacterial Systems

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in *Escherichia coli* (*E. coli*) [Raibaud *et al.* (1984) *Annu. Rev. Genet.* 18:173]. Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, 5 such as galactose, lactose (lac) [Chang et al. (1977) Nature 198:1056], and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (trp) [Goeddel et al. (1980) Nuc. Acids Res. 8:4057; Yelverton et al. (1981) Nucl. Acids Res. 9:731; U.S. 10 Patent No. 4,738,921; EPO Publ. Nos. 036 776 and 121 775]. The g-lactamase (bla) promoter system [Weissmann (1981) "The cloning of interferon and other mistakes." In Interferon 3 (ed. I. Gresser)], bacteriophage lambda PL [Shimatake et al. (1981) Nature 292:128] and T5 [U.S. Patent No. 4,689,406] 15 promoter systems also provide useful promoter sequences.

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or 20 bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter [U.S. Patent No. 4,551,433]. For example, the tac promoter is a hybrid trp-lac promoter comprised of both trp promoter and lac 25 operon sequences that is regulated by the lac repressor [Amann et al. (1983) Gene 25:167; de Boer et al. (1983) Proc. Natl. Acad. Sci. 80:21]. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA 30 polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an 35 example of a coupled promoter system [Studier et al. (1986) J. Mol. Biol. 189:113; Tabor et al. (1985) Proc Natl. Acad. Sci. 82:1074]. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an E. coli

operator region (EPO Publ. No. 267 851).

- In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In E. coli, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon [Shine et al. (1975) Nature 254:34].
- The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' end of E. coli 16S rRNA [Steitz et al. (1979) "Genetic signals and nucleotide sequences in messenger RNA." In Biological Regulation and Development: Gene Expression (ed. R.F. Goldberger)]. To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site [Sambrook et al. (1989) "Expression of cloned genes in *Escherichia coli*." In Molecular Cloning: A Laboratory Manual].
- A DNA molecule may be expressed intracellularly. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide or by either in vivo or in vitro incubation with a bacterial methionine N-terminal peptidase (EPO Publ. No. 219 237).
- Fusion proteins provide an alternative to direct expression. Usually, a DNA sequence encoding the N-terminal portion of an endogenous bacterial protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the bacteriophage lambda cell gene can be linked at the 5' terminus of a foreign gene and expressed in bacteria. The resulting fusion protein preferably retains a site for a processing enzyme (factor

Xa) to cleave the bacteriophage protein from the foreign gene [Nagai et al. (1984) Nature 309:810]. Fusion proteins can also be made with sequences from the lacZ [Jia et al. (1987) Gene 60:197], trpE [Allen et al. (1987) J. Biotechnol. 5:93; Makoff et al. (1989) J. Gen. Microbiol. 135:11], and Chey [EPO Publ. No. 324 647] genes. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated [Miller et al. (1989) Bio/Technology 7:698].

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein in bacteria [U.S. Patent No. 4,336,336]. The signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites, which can be cleaved either in vivo or in vitro encoded between the signal peptide fragment and the foreign gene.

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the E. coli outer membrane protein gene (ompA) [Masui et al. (1983), in: Experimental Manipulation of Gene Expression; Ghayeb et al. (1984) EMBO J. 3:2437] and the E. coli alkaline phosphatase signal sequence (phoA) [Oka et al. (1985) Proc. Natl. Acad. Sci. 82:7212]. As an additional example, the signal sequence of the alpha-amylase gene from various Bacillus

strains can be used to secrete heterologous proteins from B. subtilis [Palva et al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. No. 244 042].

- 5 Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the
- 10 polypeptide encoded by the DNA. Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with
- 15 strong promoters, such as the trp gene in E. coli as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of

20 interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will

25 have a replication system, thus allowing it to be maintained in a procaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to

30 about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and

35 the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector.

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Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various *Bacillus* strains integrate into the *Bacillus* chromosome (EPO Publ. No. 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

10

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline [Davies *et al.* (1978) Annu. Rev. Microbiol. 32:469]. Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

20

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable market that is either maintained in a replicon or developed into an integrating vector, as described above.

25

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For example, expression vectors have been developed for, *inter alia*, the following bacteria: *Bacillus subtilis* [Palva *et al.* (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541], *Escherichia coli* [Shimatake *et al.* (1981) Nature 292:128; Amann *et al.* (1985) Gene 40:183; Studier *et al.* (1986) J. Mol. Biol. 189:113; EPO Publ. Nos. 036 776, 136 829 and 136 907], *Streptococcus cremoris* [Powell *et al.* (1988) Appl.

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Environ. Microbiol. 54:655]; *Streptococcus lividans* [Powell et al. (1988) Appl. Environ. Microbiol. 54:655], *Streptomyces lividans* [U.S. Patent No. 4,745,056].

- 5 Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the transformation of bacteria treated with CaCl_2 or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation.
- 10 Transformation procedures usually vary with the bacterial species to be transformed. See e.g., [Masson et al. (1989) FEMS Microbiol. Lett. 60:273; Palva et al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541, *Bacillus*], [Miller et al. (1988) Proc. Natl. Acad. Sci. 85:856; Wang et al. (1990) J. Bacteriol. 172:949, *Campylobacter*], [Cohen et al. (1973) Proc. Natl. Acad. Sci. 69:2110; Dower et al. (1988) Nucleic Acids Res. 16:6127; Kushner (1978) "An improved method for transformation of *Escherichia coli* with ColE1-derived
- 20 plasmids. In Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering (eds. H.W. Boyer and S. Nicosia); Mandel et al. (1970) J. Mol. Biol. 53:159; Taketo (1988) Biochim. Biophys. Acta 949:318; *Escherichia*], [Chassy et al. (1987) FEMS Microbiol. Lett. 44:173 *Lactobacillus*]; [Fiedler et al. (1988) Anal. Biochem 170:38, *Pseudomonas*]; [Augustin et al. (1990) FEMS Microbiol. Lett. 66:203, *Staphylococcus*], [Barany et al. (1980) J. Bacteriol. 144:698; Harlander (1987) "Transformation of *Streptococcus lactis* by electroporation,
- 30 in: Streptococcal Genetics (ed. J. Ferretti and R. Curtiss III); Perry et al. (1981) Infec. Immun. 32:1295; Powell et al. (1988) Appl. Environ. Microbiol. 54:655; Somkuti et al. (1987) Proc. 4th Eur. Cong. Biotechnology 1:412, *Streptococcus*].

35

iv. Yeast Expression

Yeast expression systems are also known to one of ordinary

skill in the art. A yeast promoter is any DNA sequence capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which, if present, is usually distal to the structural gene. The UAS permits regulated (inducible) expression. Constitutive expression occurs in the absence of a UAS. Regulated expression may be either positive or negative, thereby either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter sequences. Examples include alcohol dehydrogenase (ADH) (EPO Publ. No. 284 044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EPO Publ. No. 329 203). The yeast PHO5 gene, encoding acid phosphatase, also provides useful promoter sequences [Myanohara *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:1].

In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Patent Nos. 4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory

sequences of either the ADH2, GAL4, GAL10, OR PHO5 genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (EPO Publ. No. 164 556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription. Examples of such promoters include, inter alia, [Cohen et al. (1980) Proc. Natl. Acad. Sci. USA 77:1078; Henikoff et al. (1981) Nature 283:835; Hollenberg et al. (1981) Curr. Topics Microbiol. Immunol. 96:119; Hollenberg et al. (1979) "The Expression of Bacterial Antibiotic Resistance Genes i the Yeast *Saccharomyces cerevisiae*," in: Plasmids of Medical, Environmental and Commercial Importance (eds. K>N> Timmis and A. Puhler); Mercerau-Puigalon et al. (1980) Gene 11:163; Panthier et al. (1980) Curr. Genet. 2:109;].

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide.

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See e.g., EPO Publ. No. 196 056. Another example is a ubiquitin fusion protein. Such a fusion protein is made

with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can
5 be isolated (see, e.g., PCT Publ. No. WO 88/024066).

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader
10 sequence fragment that provide for secretion in yeast of the foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either in vivo or in vitro. The leader sequence fragment usually encodes a signal peptide comprised
15 of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast
20 invertase gene (EPO Publ. No. 012 873; JPO Publ. No. 62,096,086) and the A-factor gene (U.S. Patent No. 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (EPO Publ. No. 060 057).

25 A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the
30 full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (usually about 25 to about 50 amino acid residues) (U.S. Patent Nos. 4,546,083 and 4,870,008; EPO Publ. No. 324 274). Additional leaders employing an alpha-factor leader fragment that
35 provides for secretion include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alphafactor. (See e.g., PCT Publ. No. WO 89/02463.)

Usually, transcription termination sequences recognized by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes.

10

Usually, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a procaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 [Botstein et al. (1979) Gene 8:17-24], pCl/1 [Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642-4646], and YRp17 [Stinchcomb et al. (1982) J. Mol. Biol. 158:157]. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host. See e.g., Brake et al., supra.

Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences

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flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome [Orr-Weaver et al. (1983) Methods in Enzymol. 101:228-245]. An integrating vector may
5 be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector. See Orr-Weaver et al., supra. One or more expression construct may integrate, possibly affecting levels of recombinant protein produced [Rine et al. (1983) Proc. Natl.
10 Acad. Sci. USA 80:6750]. The chromosomal sequences included in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the
15 vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the
20 selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as ADE2, HIS4, LEU2, TRP1, and ALG7, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418,
25 respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of CUP1 allows yeast to grow in the presence of copper ions [Butt et al. (1987) Microbiol. Rev. 51:351].

30 Alternatively, some of the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an
35 integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been

developed for transformation into many yeasts. For example, expression vectors have been developed for, inter alia, the following yeasts: *Candida albicans* [Kurtz, et al. (1986) Mol. Cell. Biol. 6:142], *Candida maltose* [Kunze, et al. (1985) J. Basic Microbiol. 25:141]. *Hansenula polymorpha* [Gleeson, et al. (1986) J. Gen. Microbiol. 132:3459; Roggenkamp et al. (1986) Mol. Gen. Genet. 202:302], *Kluyveromyces fragilis* [Das, et al. (1984) J. Bacteriol. 158:1165], *Kluyveromyces lactis* [De Louvencourt et al. (1983) J. Bacteriol. 154:737; Van den Berg et al. (1990) Bio/Technology 8:135], *Pichia guilliermondii* [Kunze et al. (1985) J. Basic Microbiol. 25:141], *Pichia pastoris* [Cregg, et al. (1985) Mol. Cell. Biol. 5:3376; U.S. Patent Nos. 4,837,148 and 4,929,555], *Saccharomyces cerevisiae* [Hinnen et al. (1978) Proc. Natl. Acad. Sci. USA 75:1929; Ito et al. (1983) J. Bacteriol. 153:163], *Schizosaccharomyces pombe* [Beach and Nurse (1981) Nature 300:706], and *Yarrowia lipolytica* [Davidow, et al. (1985) Curr. Genet. 10:380471 Gaillardin, et al. (1985) Curr. Genet. 10:49].

20

Methods of introducing exogenous DNA into yeast hosts are well-known in the art, and usually include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformation procedures usually vary with the yeast species to be transformed. See e.g., [Kurtz et al. (1986) Mol. Cell. Biol. 6:142; Kunze et al. (1985) J. Basic Microbiol. 25:141; *Candida*]; [Gleeson et al. (1986) J. Gen. Microbiol. 132:3459; Roggenkamp et al. (1986) Mol. Gen. Genet. 202:302; *Hansenula*]; [Das et al. (1984) J. Bacteriol. 158:1165; De Louvencourt et al. (1983) J. Bacteriol. 154:1165; Van den Berg et al. (1990) Bio/Technology 8:135; *Kluyveromyces*]; [Cregg et al. (1985) Mol. Cell. Biol. 5:3376; Kunze et al. (1985) J. Basic Microbiol. 25:141; U.S. Patent Nos. 4,837,148 and 4,929,555; *Pichia*]; [Hinnen et al. (1978) Proc. Natl. Acad. Sci. USA 75:1929; Ito et al. (1983) J. Bacteriol. 153:163 *Saccharomyces*]; [Beach and Nurse (1981) Nature 300:706; *Schizosaccharomyces*]; [Davidow et al. (1985) Curr. Genet.

10:39; Gaillardin et al. (1985) Curr. Genet. 10:49; Yarrowia].

Example 1 - Detoxified LT

5

A fragment of the gene for LT was extracted from plasmid EWD299 [Dallas W.S., Gill D.M. and Falkow S., 1979, J. Bacteriol., 139, 850-858] by digestion with the restriction enzymes SmaI and EcoRI, and was recloned in the vector
10 Bluescript KS suitable for producing single strands of DNA [Sambrook J., Fritsch E. and Maniatis, T. "Molecular Cloning", Cold Spring Harbor].

BW313 cells were transformed by the clones thus obtained and
15 allowed to grow for 14 hours in a culture medium consisting of Luria Broth with the addition of 1 µg/ml of uridine.

A series of synthetic oligonucleotides (listed in Table 1 below), containing the mutation, or the desired bases
20 instead of the natural ones, and a sequence of 10 bases upstream and 10 downstream of the same mutation, identical to the natural ones, was first of all synthesised chemically and then phosphorylated, 1.5 pmol thereof being treated at 37°C with 5 units of kinase.

25

After halting the reaction with a 100 mM EDTA solution, the oligonucleotides were annealed to the single strand containing the LT gene, by heating for 5 minutes at 70°C and cooling slowly for about one hour in ice.

30

At that stage there was added to this cold solution (25 µl) a solution of free nucleotides, the enzyme DNA ligase and the enzyme DNA polymerase, in a final volume of 100 µl.

35 The solution thus obtained was kept for five minutes in ice, five minutes at ambient temperature and two hours at 37°C.

Suitable cells of E. coli were transformed with the reaction

mixture, in accordance with the usual techniques [Sambrook J., Fritsch E. and Maniatis T. "Molecular Cloning" Cold Spring Harbor], and the site-directed mutagenesis was checked by sequencing of the clones obtained.

5

The SmaI-EcoRI fragment containing the various mutations was substituted for the original SmaI-EcoRI insert in the plasmid EWD299.

- 10 The strains which encode the mutated toxin were then grown in 10 ml of Luria Broth for 12 hours at 37°C.

The cultures were centrifuged and the precipitate containing the cells was resuspended in 300 ml of a solution containing
15 25% of sucrose and 50 mM of Tris buffer at pH8, and the mixture was treated for one hour at ambient temperature with 1 mg/ml of a solution of Polymixin B.

The presence of the toxoid in the periplasmatic supernatant
20 liquor was verified by means of Western Blot and its toxicity was evaluated by the inducement or lack of inducement of morphological changes in Y1 cells (see Table 1).

- 25 Y1 cells are adrenal tumour epithelial cells which become markedly more rounded when treated with a solution containing CT or LT [Yasamure Y., Buonassisi V. and Sato G., "Clonal analysis of differentiated function in animal cell cultures", Cancer Res., 1966, 26, 529-535]. The toxicity of
30 CT and LT is correlated with this morphological transition. The periplasmic supernatant is diluted with a solution of F10 medium, horse serum 1.5%, glutamine and gentamycin to lesser and lesser concentrations and Y1 cells (250000 cells/ml) are incubated with the resulting solutions for 48
35 hours at 37°C under an atmosphere of CO₂. The morphology of the cells is evaluated.

In all cases, immunogenicity was shown by correct assembly

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of the complete toxoid and by cross reaction of the toxoid with antibody to the wild type LT.

The results are shown in Table I below.

5

In this Table (and in Table II below) the toxicity symbols mean as follows:

- +++ toxic after dilution 1:2000 (wild type toxicity)
 10 ++ toxic up to dilution 1:250
 + toxic up to dilution 1:64
 - not toxic, even undiluted

15

TABLE I

	<u>Example</u>	<u>Mutation</u>	<u>Oligonucleotide Sequence</u>	<u>Toxicity</u>
20	1.1 LT	Val-53-Asp	291-ACCGGCTTTGATAGATATGAT-311	-
	1.2 LT	Val-53-Glu	291-ACCGGCTTTGAAAGATATGAT-311	-
	1.3 LT	Val-53-Tyr	291-ACCGGCTTTTACAGATATGAT-311	-
	1.4 LT	Ser-63-Lys	322-GTTTCCACTAAGCTTAGTTTG-342	-
	1.5 LT	Val-97-Lys	424-ATGTTTAATAAGAATGATGTA-444	-
25	1.6 LT	Val-97-Tyr	424-ATGTTTAATTACAATGATGTA-444	-
	1.8 LT	His-107-Glu	454-TACAGCCCTGAGCCATATGAA-474	+
	1.9 LT	Tyr-104-Lys	445-ATTAGCGTAAAGAGCCCT-462	-
	1.10 LT	Tyr-104-Asp	445-ATTAGCGTAGATAGCCCT-462	-
	1.11 LT	Tyr-104-Ser	447-TAGCGTAAGTAGCCCTCA-464	-
30	1.12 LT	Pro-106-Ser	453-ATACAGCAGCCACCCATA-470	-

Two mutation of serine (Ser-114-Glu:477-GGAGGTGAAGCGTTAGG-494 and Ser-114-Lys:477-GGAGGTTAAAGCGTTAGG-494) were also shown to exhibit substantially reduced toxicity.

35

Comparative Examples

	A LT	LT Wild Type		##
40	B LT	Arg-210-Asp	769-ATATATCTCAACGAATATCAA-789	+
	C LT	Leu-41-Phe	113-ATATTAATTTCTATGATC-130	NA
	D LT	His-44-Phe	121-CTTTATGATTTTGCGAGA-138	NA
	E LT	Ala-45-Tyr	125-ATGATCACTATAGAGGAA-142	NA
	F LT	Arg-54-Ala	152-GCTTTGTCGCGTATGATG-169	++
45	G LT	Arg-54-Lys	151-GGCTTTGTCAAGTATGATGAT-171	++
	H LT	Tyr-59-Met	167-ATGACGGAATGGTTTCCA-184	##
	I LT	Val-60-Gly	169-GACGGATATGGATCCACTTCT-189	NA
	J LT	Ser-68-Lys	193-AGTTTGAGAAAGGCTCACTTA-213	++
	K LT	Ser-68-Pro	193-AGTTTGAGACCAGCTCACTTA-213	NA
50	L LT	His-70-Pro	199-AGAAGTGCTCCTTTAGCAGGA-219	NA
	M LT	Ala-72-Arg	205-GCTCACTTAAGGGGACAGTCT-225	++
	N LT	Ala-72-His	205-GCTCACTTACATGGACAGTCT-225	##
	O LT	Arg-192-Asn	565-GATTCATCAATTACAATCACA-585	##

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(NA means "not assembled", i.e the holotoxin AB₅ is not formed at all)

5 Example 2 - Detoxified CT

The procedure followed in the case of the gene for the toxin CT is analogous to that described above.

- 10 A fragment containing the gene for a CT was amplified by means of the polymerase chain reaction (PCR) technique from plasmid pCT322. An alternative and equivalent source of the CT gene is plasmid pJM17 (Pearson et al, PNAS USA, 79, (1982), 2976-2980).

15

The following two synthetic primers were used:

1) GGCAGATTCTAGACCTCCTGATGAAATAAA

20 2) TGAAGTTTGGCGAAGCTTCTTAATTTGCCATACTAATTGCGGCAATCGCAT

containing respectively an XbaI site and an artificial HindIII site (shown underlined).

- 25 The resulting amplified fragment, XbaI-HindIII, which has a length of 1074 base pairs, contains the codons of the two sub-units, A and B, but not the sequence encoding the leader peptide of the A sub-unit. This fragment was recloned in Bluescript KS vector and was treated in accordance with the
- 30 procedure described above for LT, so as to effect the site-directed mutagenesis.

TABLE II

	<u>Example Mutation</u>	<u>Oligonucleotide Sequence</u>	<u>Toxicity</u>
5	2.1 CTVal-53-Asp	ACGGGATTGACAGGCACGAT	-
	2.2 CTSer-63-Lys	GTTTCCACCAAGATTAGTTTG	-
	2.3 CTVal-97-Lys	ATGTTTAACAAGAATGATGTA	-
	2.4 CTSer-106-Pro	GGCATACAGTAGCCATCCAGA	-
10	<u>Comparative Examples</u>		
	A CT Arg-192-Asn	GAATGCTCCAAACTCATCGAT	+++
	B CTArg-54-His	GGATTGTTCATCACGATGAT	++

15

The following mutations also proved to abolish toxicity: ~~H~~
 107-Asn (TACAGTCCTAACCCAGATGAA), Glu-110-Ser
 (TCATCCAGATTCGCAAGAAGT), Glu-112-Ala (CAGATGAACAAGCTGTTTCTG)
 and Ser-114-Glu (CAAGAAGTTGAAGCTTTAGGT).

20

It will be understood that the invention is described above
 by way of example only and modifications of detail may be
 made within the scope and spirit of the invention.

CLAIMS:

1. An immunogenic detoxified protein comprising the amino acid sequence of subunit A of a cholera toxin (CT-A) or a
5 fragment thereof or the amino acid sequence of subunit A of an *Escherichia coli* heat labile toxin (LT-A) or a fragment thereof wherein one or more amino acids at, or in positions corresponding to Val-53, Ser-63, Val-97, Tyr-104 or Pro-106 are replaced with another amino acid.
10
2. An immunogenic detoxified protein according to claim 1 wherein additionally one or more amino acids at, or in positions corresponding to Arg-7, Asp-9, Arg-11, His-44, Arg-54, Ser-61, His-70, His-107, Glu-110, Glu-112, Ser-114,
15 Trp-127, Arg-146 or Arg-192 are replaced.
3. An immunogenic detoxified protein according to claim 1 or 2 comprising one or more of the following amino acid replacements Val-53-Asp, Val-53-Glu, Val-53-Tyr, Ser-63-Lys,
20 Val-97-Lys, Val-97-Tyr, His-107-Glu, Tyr-104-Lys, Tyr-104-Asp, Tyr-104-Ser, Pro-106-Ser, Ser-114-Glu, Ser-114-Lys.
4. An immunogenic composition for use as a vaccine comprising an immunogenic detoxified protein according to
25 any one of the preceding claims and a pharmaceutically acceptable carrier.
5. A vaccine composition comprising an immunogenic detoxified protein according to any one of claims 1 to 3 and
30 a pharmaceutically acceptable carrier.
6. A vaccine composition according to claim 5 further comprising an adjuvant.
- 35 7. A DNA sequence encoding an immunogenic detoxified protein according to any one of claims 1 to 3.
8. A vector carrying a DNA according to claim 7.

50

9. A host cell line transformed with the vector according to claim 8.
- 5 10. A process for the production of an immunogenic detoxified protein according to any one of claims 1 to 3 comprising culturing a host cell according to claim 9.
- 10 11. A process for the production of a DNA according to claim 7 comprising the steps of subjecting a DNA encoding a CT-A or an LT-A or a fragment thereof to site-directed mutagenesis.
- 15 12. A method of vaccinating a mammal against *Vibrio cholerae* or an enterotoxigenic strain of *Escherichia coli* comprising administering an immunologically effective amount of an immunogenic detoxified protein according to any one of claims 1 to 3.
- 20 13. A process for the formulation of a vaccine according to claim 5 comprising bringing an immunogenic detoxified protein according to any one of claims 1 to 3 into association with a pharmaceutically acceptable carrier.
- 25 14. A process for the formulation of a vaccine according to claim 6 comprising bringing an immunogenic detoxified protein according to any one of claims 1 to 3 into association with an adjuvant.

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LT2	1	-.-.FF-----T----R-A---L---QQ-AYE---PI----	38
LT1	1	-----FRS-----	39
LT1_1A	1	-G-R-----R-----HN-----	40
CT	1	NDDKLYRADSRPPDEIKQSGGLMPRGQSEYFDRGTQMNIN	40

--E-----V--NT--N-----TVT--Q---I--N--GS-	78
-----Y-----	79
-----Y-----L-----A--S---Y	80
LYDHARGTQTGFVRHDDGYVSTSLSLRSAHLVGQTILSGH	80

NE-----V-P---L-D--G---R---Y-S-N-FA-----	118
-LTIYI---...-----IS-----	116
-----V-----Y-----	120
STYVIYVIATAPNMFNVNDVLGAYSHPDEQEVSALGGIP	120

L---I-----SF-A-EGGMQ---D--GDLF-G-TV--N--	158
-----	156
-----N---I--R-----E-----R--N---E-	160
YSQIYGWYRVHFGVLDEQLHRNRYRDRYYSNLDIAPAAD	160

--Q-----SNFP-----M--STF--EQ-VPNNKEFK-GV-I	198
-----	196
--R-----D-Q-----Q---DSS-TITGD--N	200
GYGLAGFPPEHRAWREEPWIHHAPPGCGNAPRSSMSNTCD	200

SA-NV--KYD-MNFKKLL--RLALTFFM--D-F-GVHGE----	241
-----	236
-E--N-STIY-R-----D-.--EV-.IY---.R---	240
EKTQSLGVKFLDEYQSKVKRQIFSGY.QSDID.THNRI.KDEL	240

Figure 1

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```

LT  AATGGCGACAGATTATACCGTGCTGACTCTAGACCCCCAGATGAAATAAAACGTTTCCGG
    N G D R L Y R A D S R P P D E I K R F R      20
-----
    N D D K L Y R A D S R P P D E I K Q S G      20
CT  AATGATGATAAGTTATATCGGGCAGATTCTAGACCTCCTGATGAAATAAAGCAGTCAGGT

LT  AGTCTTATGCCCAGAGGT...AATGAGTACTTCGATAGAGGAACTCAAATGAATATTAAT
    S L M P R G Q N E Y F D R G T Q M N I N      39
-----
    G L M P R G Q S E Y F D R G T Q M N I N      40
CT  GGTCTTATGCCAAGAGGACAGAGTGAGTACTTTGACCGAGGTACTCAAATGAATATCAAC

LT  CTTTATGATCACGCGAGAGGAACACAAACCGGCTTTGTGATATGATGACGGATATGTT
    L Y D H A R G T Q T G F V R Y D D G Y V      59
-----
    L Y D H A R G T Q T G F V R H D D G Y V      60
CT  CTTTATGATCATGCAAGAGGAACTCAGACGGGATTTGTTAGGCACGATGATGGATATGTT

LT  TCCACTTCTCTTAGTTTGTAGAAGTGCTCACTTAGCAGGACAGTATATATTATCAGGATAT
    S T S L S L R S A H L A G Q Y I L S G Y      79
-----
    S T S I S L R S A H L V G Q T I L S G H      80
CT  TCCACCTCAATTAGTTTGTAGAAGTGCCCACTTAGTGGGTCAAACCTATATTGTCTGGTCAT

LT  TCACTTACTATATATATATCGTTATAGCA.....AATATGTTTAAATGTTAATGATGTA
    S L T I Y I V I A          N M F N V N D V      96
-----
    S T Y Y I Y V I A T A P N M F N V N D V      100
CT  TCTACTTATTATATATATATGTTATAGCCACTGCACCCAACATGTTTAAACGTTAATGATGTA

LT  ATTAGCGTATACAGCCCTCACCCATATGAACAGGAGGTTTCTGCGTTAGGTGGAATACCA
    I S V Y S P H P Y E Q E V S A L G G I P      116
-----
    L G A Y S P H P D E Q E V S A L G G I P      120
CT  TTAGGGGCATACAGTCCTCATCCAGATGAACAAGAAGTTTCTGCTTTAGGTGGGATTCCA

```

Figure 2a

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LT TATTCTCAGATATATGGATGGTATCGTGTTAATTTTGGTGTGATTGATGAACGATTACAT
Y S Q I Y G W Y R V N F G V I D E R L H 136

Y S Q I Y G W Y R V H F G V L D E Q L H 140
CT TACTCCCAAATATATGGATGGTATCGAGTTCATTTTGGGGTGCTTGATGAACAATTACAT

LT CGTAACAGGGAATATAGAGACCGGTATTACAGAAATCTGAATATAGCTCCGGCAGAGGAT
R N R E Y R D R Y Y R N L N I A P A E D 156

R N R G Y R D R Y Y S N L D I A P A A D 160
CT CGTAATAGGGGCTACAGAGATAGATATTACAGTAACCTAGATATTGCTCCAGCAGCAGAT

LT GGTACAGATTAGCAGGTTTCCACCGGATCACCAAGCTTGGAGAGAAGAACCCTGGATT
G Y R L A G F P P D H Q A W R E E P W I 176

G Y G L A G F P P E H R A W R E E P W I 180
CT GGTATGGATTGGCAGGTTTCCCTCCGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATT

LT CATCATGCACCACAAGGTTGTGGAGATTCATCAAGAACAATCACAGGTGATACTTGTAAT
H H A P Q G C G D S S R T I T G D T C N 196

H H A P P G C G N A P R S S I S N T C D 200
CT CATCATGCACCGCCGGGTTGTGGGAATGCTCCAAGATCATCGATCAGTAATACTTGCGAT

LT GAGGAGACCCAGAATCTGAGCACAATATATCTCAGGGAATATCAATCAAAAGTTAAGAGG
E E T Q N L S T I Y L R E Y Q S K V K R 216

E K T Q S L G V K F L D E Y Q S K V K R 220
CT GAAAAAACCCTAGGTGTAAATTCCTTGACGAATACCAATCTAAAGTTAAAGA

LT CAGATATTTTCAGACTATCAGTCAGAGGTTGACATATATAACAGAATTCGGGATGAATTATGA
Q I F S D Y Q S E V D I Y N R I R D E L *

Q I F S G Y Q S D I D T H N R I K D E L *
CT CAAATATTTTCAGGCTATCAATCTGATATTGATACACATAATAGAATTAAGGATGAATTATGA

Figure 2b

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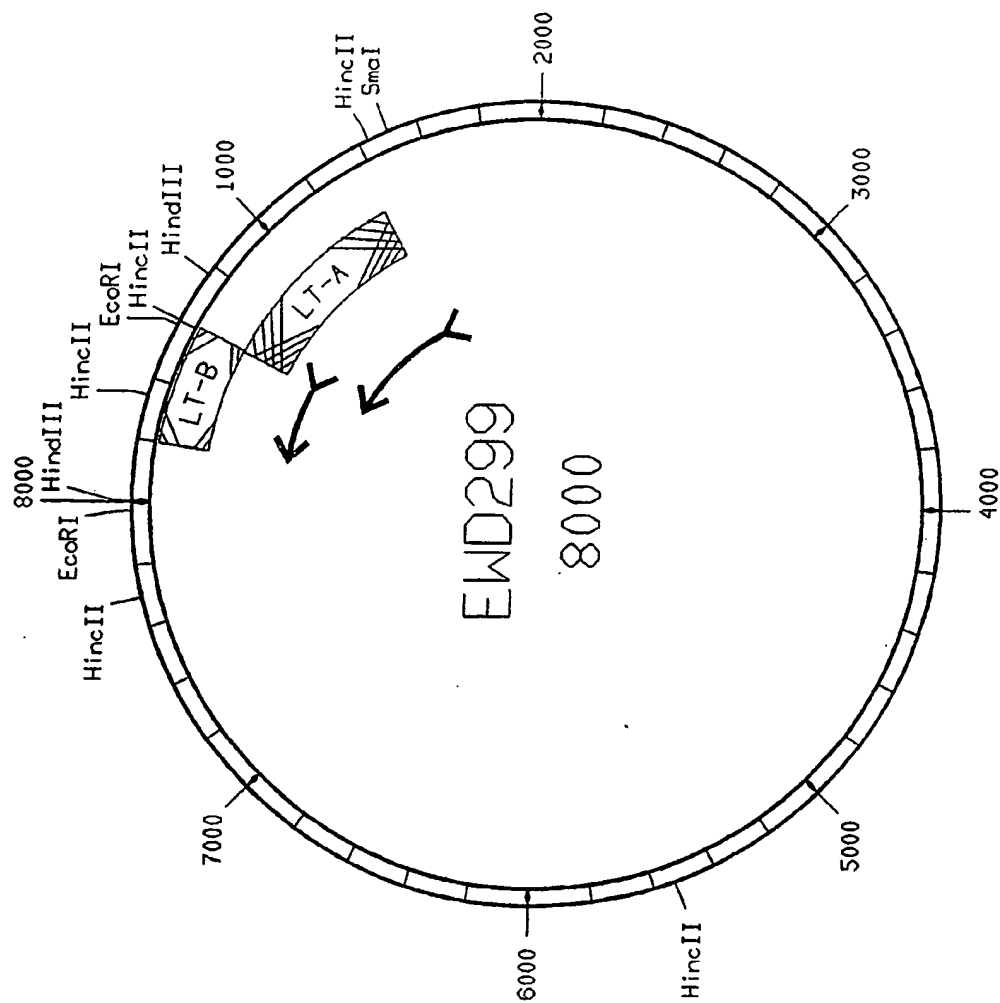


Figure 3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 92/03016

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/00; C12P21/02;	C12N15/31; C12N1/21;	A61K39/106; /(C12N1/21,C12R1:19) A61K39/108
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C12P ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	<p>INFECTION AND IMMUNITY vol. 59, no. 9, September 1991, AM. SOC. MICROBIOL., BALTIMORE, US; pages 2870 - 2879 Y. LOBET ET AL. 'Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the a subunit of Escherichia coli heat-labile enterotoxin' cited in the application see page 2871, right column, line 7 - page 2873, right column, line 5</p> <p style="text-align: center;">---</p> <p style="text-align: right;">-/--</p>	1,2,7,11
<p>¹⁰ Special categories of cited documents : ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
20 APRIL 1993		10. 05. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		HORNIG H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category ^o	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>INFECTION AND IMMUNITY vol. 59, no. 11, November 1991, AM. SOC. MICROBIOL., BALTIMORE, US; pages 4266 - 4270 W.N. BURNETTE ET AL. 'Site-specific mutagenesis of the catalytic subunit of cholera toxin: Substituting lysine for arginine 7 causes loss of activity' cited in the application see page 4266, right column, line 14 - line 29</p> <p style="text-align: center;">---</p>	1,2,7,11
Y	<p>J. BACTERIOLOGY vol. 170, no. 5, May 1988, AM. SOC. MICROBIOL., BALTIMORE, US; pages 2208 - 2211 K. OKAMOTO ET AL. 'Effect of substitution of glycine for arginine at position 146 of the A1 subunit on biological activity of Escherichia coli heat-labile enterotoxin' see page 2208, right column, line 18 - page 2209, left column, line 7</p> <p style="text-align: center;">---</p>	1,2,7,11
P,Y	<p>WO,A,9 219 265 (AMGEN INC.) 12 November 1992 cited in the application see page 22, line 25 - page 27, line 26</p> <p style="text-align: center;">-----</p>	1,2,7,11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 92/03016

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 12 is directed to a method of treatment of the human /animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

EP 9203016
SA 69235

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9219265	12-11-92	AU-A- 2293092	21-12-92



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| EP-A- 0 020 147 | EP-A- 0 049 945 |
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| WO-A-83/02393 | WO-A-83/03971 |
| WO-A-85/04654 | WO-A-85/05037 |
| AU-A- 1 199 083 | AU-A- 3 449 784 |
| AU-A- 4 458 885 | AU-B- 1 470 576 |
| FR-A- 2 306 684 | GB-A- 2 071 671 |
| GB-A- 2 094 314 | GB-B- 1 529 871 |
| US-A- 4 372 883 | US-A- 4 496 538 |
| US-A- 4 556 555 | |

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EP 0 222 835 B1

Journal of Immunological Methods, Vol. 48
Issued 1982 (Amsterdam, Netherlands)
P.R.Lambden and J.E. Heckels, pages
233-240

Progress In Allergy, vo. 33, Issued 1983 (Ba-
sel, Switzerland) Schoolnik et al, "A Pilus,
Peptide Vaccine for the Prevention of Gon-
orrhea", see pages 314-331

M. Susman, "The Virulence of Escherichia
coli" 1985, Academic Press, see pages
82-37, 111-119

In another aspect of this invention a process is described whereby a hapten or protein can be coupled to a mucosal immunogen and the complex of which, when fed, results in the production of antibodies to the hapten or coupled protein.

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ABBREVIATIONS		
1.	Ab	Antibody
2.	BSA	bovine serum albumin
3.	ConA	Concanavalin A
4.	DNP	dinitrophenyl
5.	ELISA	Enzyme linked immunosorbent assay
6.	ETEC	enterotoxigenic <i>E. coli</i>
7.	GALT	gut associated lymphoid tissue
8.	HA	hydroxy apatite
9.	im	intra muscular
10.	LHRH	luteinizing hormone releasing hormone.
11.	LPS	lipopolysaccharide
12.	LT-B	heat labile toxin of enterotoxigenic <i>E. coli</i> .
13.	O/N	overnight
14.	per oo	oral administration
15.	ps	polysaccharide
16.	RT	room temperature
17.	sc	subcutaneous
18.	SDS-PAGE	SDS - polyacrylamide gel electrophoresis
19.	TCA	trichloroacetic acid.

DISCLOSURE OF INVENTION

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In a first form the invention provides a complex comprising: an immunogen suitable for vaccination purposes;

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linked to a carrier molecule which is capable of specifically interacting with the mucosal epithelium of a vertebrate host; wherein both the immunological activity of the immunogen and the capacity of the carrier molecule to specifically interact with the mucosal epithelium of the vertebrate host is substantially maintained, and said complex is capable of eliciting a systemic, cellular and/or mucosal immune response in the vertebrate host.

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Preferred immunogens according to the invention include: an antigen or hapten or all, part, analogues, homologues, derivatives or combinations of a hormone or therapeutic agent. These immunogens include hormones such as LHRH (luteinising hormone releasing hormone) FSH, HGH and Inhibin; allergens such as grass pollens (for instance barley and couch), weed pollens (eg. clover, dock), tree pollens (eg. ash, cyprus), plant pollens (eg. broom), epithelia (eg. cat hair, dog hair, pig hair) and house dust, wheat chaff and Kapok; immunogens for vaccines against agents such as influenza, measles, Rubella, smallpox, yellow fever, diphtheria, tetanus, cholera, plague, typhus, BCG, *haemophilus influenzae*, *Neisseria catarrhalis*, *Klebsiella pneumoniae*, *pneumococci* and *streptococci* especially *S. mutans*; and pili including pili derived from *E. coli*, *N. gonorrhoeae*, *N. meningitis*, *N. catarrhalis*, *Yersinia* spp, *Pseudomonas aeruginosa*, *Pseudomonas* spp, *Moraxella bovis*, *Bacteroides nodosus*, *Staphylococci* spp, *Streptococci* spp and *Bordetella* spp.

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Preferred carrier molecules include bacterial adhesins such as 987P, K99, CFAI, CFAll, K88 or F41; viral haemagglutinins such as from influenza, measles, Rubella, smallpox or yellow fever viruses; toxins or binding subunits thereof such as LTB ricin, abrin, diphtheria toxin, modecin, tatanus toxcin and others of similar structures; and lectins whether from plant or other origin. Lectins include for example concanavalin A, Pokeweed mitogen or lectins from *Lens culinaris*, *Helix pomatia*, *Glycine max*, *Arachis hypogea*, or *Ulex europeus* or Abrin, Asparagus pea, Broad bean, Camel's foot tree, Castor bean, Fava bean, Green marine algae, Hairy vetch, Horse gram, Horse shoe crab, Jack bean, Japanese wisteria, Jequirity, Scotch laburnum, Lima bean, Limulin, Lotus, European mistletoe, Mung bean, Osage orange, Pagoda tree, Garden pea, Potato, Red kidney bean, Red marine algae, Siberian pea tree, edible snail, garden snail, Spindle tree, Sweet pea, Tomato, wheat germ or winged pea.

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Preferably the polynucleotide sequence is one wherein the first hybrid polynucleotide sequence acts as a coding sequence for the amino acid sequence of all, part, an analogue, homologue, derivative or combination thereof of LHRH fused to the amino acid sequence of a carrier molecule, more preferably LTB.

In a further form of the invention there is provided a medicament which comprises a complex according to the invention together with a pharmaceutically acceptable carrier or diluent. Examples of pharmaceutically acceptable carriers and diluents include typical carriers and diluents such as tablets, aqueous solutions, sodium bicarbonate solutions and similar diluents which neutralise stomach acid or have similar buffering capacity, glycols, oils, oil-in-water or water-in-oil emulsions, and include medicaments in the form of emulsions, gels, pastes and viscous colloidal dispersions. The medicament may be presented in capsule, tablet, slow release or elixir form or as a gel or paste or may be presented as a nasal spray and in this form may be in the presence of an aerosol. Furthermore, the medicament may be provided as a live stock feed or as food suitable for human consumption.

The present inventors have also found that co-administration of certain dietary molecules with a complex of the present invention can selectively modulate the magnitude and/or type of the immune response to the immunogen of the complex.

Accordingly the present invention further provides a medicament which comprises the complex of the present invention together with a dietary molecule which dietary molecule can selectively modulate the magnitude and/or type of the immune response to the immunogen of the complex.

The dietary molecule envisaged by the present invention include basic, neutral and acidic amino acids, such as arginine, histidine, lysine, alanine, cysteine, cystine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, aspartic acid, glutamic acid; water soluble and insoluble vitamins, such as thiamine, riboflavin, pyridoxal cyanocobalamin (V.B₁₂) ascorbic acid (V.C). Vit D₂, etc - Ergosterol, Vit.E, Vit.A, Vit K etc; sugars including monosaccharides e.g. galactose, mannose, mannitol, sorbitol, glucose, xylose, Allose, altrose, arabinose, digitoxose, erythrose, fructose, lyxose, muramic acid, mannose, pyruvic acid, ribose, tagatose, talose and the amidated and N acetylated derivatives thereof; oligosaccharides e.g. lactose, maltose, melibiose, sucrose, cellubiose, N,N diacetyl chitobiose, gentobiose, isomaltose, lactobionic acid, trehalose, turanose; and dietary minerals and co-factors such as manganese, magnesium, zinc, calcium and iron.

The invention also provides a method of presenting a complex of the present invention which method comprises the mucosal administration of a complex of the present invention together with a dietary molecule capable of modulating the magnitude and/or type of immune response of the immunogen.

The invention also provides the oral administration of the medicaments of the invention, in order to elicit a response to the active molecule in the host. Such a response, in the case where the active molecule is an antigen or hapten may be a systemic and/or a mucosal immune response. In the case where the active molecule is LHRH or a derivative, analogue, homologue, part or combination thereof, of LHRH, the response will be inhibition of gonadal function in the host. Where the oral medicament incorporates a dietary molecule according to the invention, the invention provides a method for enhancing the host's response to the active molecule which comprises administering such an oral medicament to the host.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the N-terminal amino acid sequence of the 987P pilin subunit, in comparison with the N-terminal amino acid sequence of other pilin proteins.

MODES FOR CARRYING OUT THE INVENTION

Materials

Lectins were purchased from Sigma Chemical Co. Inactivated flu vaccine was purchased from the Commonwealth Serum Labs. (Australia). Sugars and vitamins were obtained from the following sources:- Lactose (AR grade) - Ajax Chemicals, Sydney, Australia; Fructose D(-), Mannose D(+), Sorbitol and Xylose D(+) (all AR grade)- B.D.H. Chemicals Ltd. Poole, England; Melibiose D(+)- Sigma Chemical Co., St. Louis, Miss.; Retinal (Vit A. aldehyde)- Fluka AG, Chemicals, Fabrik Buchs, Switzerland; Thiamine - HCl (Vit B1), Riboflavin (Vit B2), Pyridoxal (Vit B6), Cyanocobalamin (Vit B12), L-ascorbic acid (Vit C), Ergosterol (Pro Vit D) and dl-a-tocopherol (Vit E)- Sigma Chemical Co., St. Louis, Miss.

prior to loading on the ion-exchange columns equilibrated with the same buffers. K99 and 987P were loaded onto CM and DEAE columns respectively at a flow rate of 100ml/hr, washed with 2 volumes of loading buffer and the pili eluted using a linear gradient from 10 mM to 0.5 M NaCl in the equilibration buffers. Fractions were examined by SDS-PAGE for protein content and LPS contamination, according to the method of Tsai and Frasch (1982).

LTB Purification

Three litres of LTB supernatant was diluted to 6l with dH₂O. The pH was adjusted to 6.5 with glacial acetic acid and loaded onto a 5 x 30 cm column of fastflow CM-Sepharose equilibrated with 10 mM phosphate buffer pH 6.5 at a flow rate of 1.2 l/hr. The column was then washed with 400 mls of 10 mM phosphate buffer pH 6.5 and bound protein eluted with a linear gradient of 10-500 mM NaCl in 10 mM phosphate pH 6.5. Fractions were collected and analysed by SDS-PAGE, the LTB peak was pooled.

Flagellae Isolation

Late log phase cultures of bacteria were pelleted by centrifugation (3,000 x g for 15 mins. at 4°C). The cells were resuspended in saline and heated at 60°C for 30 minutes, followed by centrifugation (3000 x g, 10 min 4°C). The supernatant was precipitated by adding a solution of 100% TCA (w/v) to give a final concentration of 10% (w/v) and spun for 10 min at 1,500 x g 4°C. The pellet was resuspended in a small volume of 1 M Tris pH 8.8 and sonicated until in solution. Ethanol was added to a final concentration of 80% (v/v) and the flagellae spun down at 2,000 x g, 10 min at 4°C. The pellet was resuspended in acetone, sonicated into suspension and reprecipitated by centrifugation (5,000 x g). Finally the pellet was brought into solution by boiling in 10% SDS and 50 mM EDTA in 10 mM Tris. HCl pH 8.0. prior to Sephacryl S-200 chromatography.

Flagellae purification

After boiling for 15 min the flagellae were clarified by centrifugation for 5 min. in a Beckman benchtop microfuge to remove non-solubilized material. The supernatant was applied to a 2.5 x 80 cm column of Sephacryl - S200 (Pharmacia, Fine Chemicals) equilibrated with 20 mM Tris pH 8.8, 0.1% SDS and 10 mM EDTA and eluted using the same buffer. Fractions were collected and analysed by SDS-PAGE. Finally the flagellae peak was pooled and precipitated with 10% (final conc.) TCA followed by centrifugation, ethanol and acetone washes as described previously. The final pellet was resuspended in dH₂O.

Lipopolysaccharide (LPS) Purification

Overnight cultures of *S. typhimurium* were extracted (30 min R.Temp) with 0.5 M CaCl₂ in 20% ethanol (v/v) containing 100 mM citrate pH 3.0 and 5% Zwittergent 3,12 (w/v) (Calbiochem.). Bacteria were pelleted by centrifugation (3,000 x g, 10 min at 4°C) and the pellet resuspended in 50 mM EDTA pH 8.0. The suspension was stirred vigorously for 30 min at R.T. After removal of the bacteria by centrifugation ethanol was added to the supernatant to a final concentration of 75%. Protein material was pelleted and the supernatant adjusted to 90% ethanol. The precipitate which formed was pelleted and washed with acetone, reprecipitated and finally resuspended in dH₂O. The preparation was assayed for sugar content using the Anthrone reagent (Herbert et al, 1985) and checked for the presence of contaminating proteins using SDS-PAGE. Commercial *E. coli* LPS (Sigma Chemical Co.) was used as a standard in both assays. Gels were stained for LPS using a silver stain according to the method of Tsai and Frasch (1982).

Preparation of Polysaccharide (PS)

Lipid A was cleaved from the *S. typhimurium* LPS preparation by incubating the LPS with 1 M glacial acetic acid and heating at 100°C for 2-5 hrs. Lipid A was then removed by centrifugation at 3,000 x g for 10 mins at 4°C.

Description of Purified Antigens

SDS-PAGE analysis of purified K99 and 987P pili preparations revealed the presence of a single band migrating at 17,500 and 20,000 mol. wt. (respectively) under reducing conditions (Fig. 1). This agrees with

Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA for the determination of antibody titres was performed as described previously by Russell-Jones et al., (1984).

EXAMPLE 1Identification of Molecules Active as Mucosal Immunogens

The possible potential of a number of molecules known to possess the capacity to bind to the intestinal mucosa and to stimulate the production of an immune response after oral administration of these molecules was examined. The response generated by these molecules was compared to the response seen after similar feeding of other molecules having no mucosal binding functions.

As seen in Table 1.1, three broad classes of proteins were detected in these experiments:- those that elicited a serum and intestinal response, K99, 987P, LTB, flu vaccine and the various lectins (Class I) (these shall henceforth be referred to as mucosal immunogens), those that elicited only a serum response (LPS) (Class II) and those that failed to elicit either a serum or intestinal response at the doses tested (Flagellae, BSA and P.S.) (Class III). Within class I antigens 987P was a significantly superior stimulator of IgA antibody (ab) (48.5 ± 1.8) when compared to LTB (12.2 ± 4.4), or K99 (3.2 ± 4.9). In addition 987P also stimulated gastrointestinal IgG (10.8 ± 1.76) to a greater extent than either K99 (3.0 ± 5.3), or LTB (1.0), and only 987P was capable of stimulating serum IgA (10.8 ± 8.8). All four class I antigens stimulated serum IgG to similar degrees (Table 1.1). The class II antigen, LPS, stimulated a small serum IgG response (12.1 ± 1.0) with no concomitant IgA or gastrointestinal reactivity. Finally BSA, flagellae and the polysaccharide moiety of LPS - class III antigens - failed to induce either serum or intestinal IgG or IgA. Representative samples from all three classes; K99, 987P, LTB, LPS and flagellae were administered intramuscularly and screened for both serum and intestinal response (Table 1.2). Class I and II antigens gave similar serum IgG responses yet failed to produce serum IgA or intestinal IgG/IgA Ab responses. Only the anti-LPS serum IgG response appeared significantly improved by i.m. immunisation. Each of 987P, K99 and LTB were further examined in dose response studies after both oral and i.m. administration. As seen in Tables 1.3 and 1.4, 987P yielded consistently higher titers than either K99 or LTB regardless of the route of administration. Interestingly the class I antigen - LTB - displayed a bell shaped dose-response with a plateau maximum between 10 and 50 μ g. None of the other class I antigens produced this effect, nor did the LTB when administered Im. Oral administration of all class I antigens (Ag) elicited higher levels of intestinal IgA Ab (S-IgA) over the broad range of doses tested. Comparison between the two routes of administration suggests that although i.m. injection consistently gave higher titers, oral administration of mucosal immunogens resulted in comparable levels of antibody production to that obtained by the i.m. route between 10 and 100 μ g of antigen for class I and II antigens.

Table 1.2

Immune reactions to antigens presented intramuscularly

Antigen used for immunisation (20 µg does)	Immune response,* day 21.			
	Serum		Intestinal	
	IgG	IgA	IgG	IgA
K99	1024 ± 94	<4	<4	<4
987P	1552 ± 112	<4	<4	<4
LTB	1782 ± 100	<4	<4	<4
Flagellae	1595 ± 227	<4	<4	<4
LPS	388 ± 58	<4	<4	<4

* See Table 1.1

Table 1.3

Dose response to orally presented antigens						
Antigen	Immune response*, day 21, per dose (µg)					
	0.1	1.0	10	50	100	1,000
Serum IgG						
K99	1.0	4.0	28	675	1351	4096
987P	4.0	14	84	588	1024	3104
LTB	9.0	194	1351	1331	891	891
Serum IgA						
K99	<4	<4	<4	<4	<4	<4
987P	<4	<4	<4	9.6	18.3	32
LTB	<4	<4	<4	<4	<4	<4
Intestinal IgG						
K99	<4	<4	<4	<4	<4	<4
987P	<4	<4	<4	4.0	4.0	8.0
LTB	<4	<4	<4	<4	<4	<4
Intestinal IgA						
K99	<4	<4	<4	4.0	16.7	87
987P	<4	<4	9.1	54	84	147
LTB	<4	<4	<4	4.0	4.0	8.0

* see Table 1.1.

structure of these compounds to Galactose which is claimed to be the specific sugar determinant on the GM1 ganglioside to which LTB is known to bind. These results are broadly suggestive that K99, 987P and LTB bind to and are internalized by discrete cells of the microvillous epithelium.

Dose response experiments (Tables 2.4, 2.5 and 2.6) demonstrate that it is possible to stimulate the secretory arm of the immune system without concomitant stimulation of serum antibodies, or conversely to augment the serum response without affecting the level of secretory Abs, by the simple addition of dietary molecules to the orally presented mucosal immunogens. Thus cofeeding of large doses of Vit B12 or melibiose with K99 leads to a two to eightfold (respectively) increase in serum Ab with little concomitant increase in secretory Abs. Conversely cofeeding of Vit D in increasing doses lead to a drop in serum Abs and a rise in secretory Ab. Certain dietary molecules on the other hand also result in stimulation of both secretory and serum Ab titres as shown by an eightfold increase in serum Ab and a 1000 fold increase in S-IgA upon cofeeding of Vit C with 987P.

Experiments in which the mucosal immunogens were injected i.m. together with vitamins or sugars showed little effect if any on the immune response thus demonstrating that the change in response due to cofeeding of these molecules with the mucosal immunogens must occur on or near the site of absorption of these molecules rather than directly upon the immune system (Table 2.7).

TABLE 2.1

Effects of dietary molecules on the immune response to orally administered K99 (20 μ g)*					
Dietary Molecule	Dose	Antibody Response			
		Serum		Intestinal	
		IgG	IgA	IgG	IgA
none	-	968 \pm 120	<4	3.0 \pm 5.2	3.2 \pm 4.9
Vit A	20 μ g	278 \pm 184	3.4 \pm 4.7	1.5 \pm 1.1	5.4 \pm 3.0
Vit B1	"	117 \pm 107	1.5 \pm 2.0	2.7 \pm 1.0	2.1 \pm 0.9
Vit B2	"	604 \pm 216	<4	2.3 \pm 1.7	2.0 \pm 0.6
Vit B6	"	14 \pm 50	<4	2.0 \pm 1.5	3.5 \pm 8.2
Vit B12**	"	3377 \pm 1266	4.0 \pm 3.0	<4	<4
Vit C**	"	318 \pm 255	2.0 \pm 2.8	32 \pm 1.1	98 \pm 70
Vit D**	"	1921 \pm 640	<4	<4	6.3 \pm 2.7
Vit E	"	512 \pm 128	<4	<4	4.4 \pm 2.1
Fructose	50mM	1782 \pm 966	8.4 \pm 3.7	2.9 \pm 1.6	34.7 \pm 14.2
Lactose	"	84 \pm 204	<4	<4	22.9 \pm 6.7
Mannose	"	1176 \pm 411	2.6 \pm 3.4	10.2 \pm 3.4	21.1 \pm 40.3
Melibiose**	"	1840 \pm 208	1.2 \pm 1.4	3.2 \pm 2.0	4.4 \pm 3.7
Sorbitol	"	77 \pm 179	<4	1.3 \pm 0.4	20.5 \pm 3.4
Xylose	"	328 \pm 217	<4	1.9 \pm 1.1	2.8 \pm 1.3

* The reciprocal of the antiserum dilution that gave an ELISA reading of 0.5 after 45 min at 37 ° C on day 21 after immunisation. Each value is the mean of five mice \pm 1 standard deviation.

** Each value is the mean of 15 mice \pm 1 standard deviation. These molecules were also tested in dose response experiments.

TABLE 2.4

5	Effect of orally administered dietary molecules on the immune response to oral antigen (K99)*							
	Dietary Molecule	Day 21 Immune response per dose (μ g ~, mM +)						
		0.1	1.0	10	50	100	500	1000
10	Serum IgG							
	Vit B12	256	675	1176	1552	2352	nd	2352
	Vit C	512	891	588	675	1024	nd	891
	Vit D	588	588	776	891	1782	nd	891
	Melibiose	128	256	675	4096	9410	256	nd
15	Serum IgA							
	Vit B12	<4	<4	4.0	5.2	<4	nd	<4
	Vit C	<4	<4	4.0	16.0	16.0	nd	16.0
	Vit D	<4	<4	<4	<4	<4	nd	<4
	Melibiose	<4	<4	<4	4.0	4.0	4.0	nd
20	Intestinal IgG							
	Vit B12	<4	<4	<4	<4	<4	nd	<4
	Vit C	<4	4.0	16.2	32.0	36.1	nd	38.0
	Vit D	<4	<4	<4	<4	<4	nd	<4
	Melibiose	<4	<4	<4	4.0	4.0	4.5	nd
25	Intestinal IgA							
	Vit B12	<4	<4	<4	<4	5.2	nd	13.9
	Vit C	<4	<4	4.0	16.0	36.7	nd	73.5
	Vit D	<4	<4	4.0	8.0	13.9	nd	24.2
	Melibiose	5.2	4.0	4.0	4.0	9.2	4.0	nd

~, dose in μ g, vitamins;

+, dose in mM, sugars.

* See Table 2.1

TABLE 2.6

Effect of orally administered dietary molecules on the immune response to oral antigen (LTB)*							
Dietary Molecule	Day 21 Immune response per dose (ug -,mM +)						
	0.1	1.0	10	50	100	500	1000
Serum IgG							
Vit B6	2352	5048	55.7	10.5	4.6	nd	4.0
Lactose	1782	1782	73.5	6.9	4.0	4.0	nd
Sorbitol	1024	256	18.3	13.9	4.0	4.0	nd
Serum IgA							
Vit B6	<4	<4	<4	<4	<4	nd	<4
Lactose	<4	<4	<4	<4	<4	<4	nd
Sorbitol	<4	1.4	<4	<4	<4	<4	nd
Intestinal IgG							
Vit B6	<4	<4	<4	<4	<4	nd	<4
Lactose	<4	<4	<4	<4	<4	<4	nd
Sorbitol	<4	<4	<4	<4	<4	<4	nd
Intestinal IgA							
Vit B6	5.6	<4	<4	<4	<4	nd	<4
Lactose	4.0	4.0	<4	<4	<4	<4	nd
Sorbitol	4.0	<4	<4	<4	<4	<4	nd

-, dose in ug, vitamins;
 +, dose in mM, sugars
 * see Table 2.1

TABLE 2.7

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Effect of varying the dose of co-administered dietary molecules on the immune response to intramuscularly presented antigen K99, 987P*							
Antigen given	Dietary molecule	Day 21 Immune response					
	(ug)	0.1	1.0	10	50	100	1000
(s) IgG							
K99	Vit B12	1121	1468	1572	2328	4766	2109
K99	Vit D	1024	1272	1168	1372	1489	1315
987P	Vit C	1687	1529	1707	1700	1662	1891
	(mM)	0.1	1.0	10	50	100	500
(s) IgG							
K99	Melibiose	1024	1176	1057	1392	1262	989
987P	Melbiose	1538	1622	1701	1519	1666	1621
987P	Sorbitol	1670	1577	1548	1632	1711	1651

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+ . no serum IgA, intestinal IgG or intestinal IgA was detected

* See Table 2.1

TABLE 3.1

5	Antibody response to DNP-modified mucosal immunogens.					
	Immunogen	Dose (ug)	Immune response*			
			Anti-DNP		Anti-Carrier	
			serum IgG	Int IgA	serum IgG	Int IgA
10	K99	20	<4	<4	875±62	3.9±5.1
	K99	100	<4	<4	1351±128	16.7±2.3
	DNP6.K99	20	21±10.5	<4	64±7.2	<4
	DNP18.K99	500	1024±77	42±9.6	128±27.4	76±12.9
	DNP1.8.K99	500	1176±164	28±14.4	3565±192	88±21.0
15	987P	20	<4	<4	891±76	27.8±13.6
	987P	100	<4	<4	1024±89	84±22.4
	DNP6.987P	20	24±3.1	<4	147±12.2	<4
	DNP25.987P	500	1024±244	14±3.1	111±34.1	68±19.2
	DNP2.5.987P	500	1351±196	7±1.4	2048±166	128±38.4
20	LTB	20	<4	<4	1351±211	12.2±4.4
	DNP2.3.LTB	20	24.3±5.6	<4	445±35	<4
	LTB	100	<4	<4	891±56	4.0
	DNP6BSA	20	<4	<4	<4	<4
	DNP6BSA	100	<4	<4	<4	<4
25	Con A™	20	666±84	<4	nd	nd
	PW-mitogen™	20	641±119	<4	nd	nd
	L. culinaris™	20	954±48	<4	nd	nd
	H. pomatia™	20	591±127	<4	nd	nd
	P. vulgaris™	20	1378±110	4.8±2.3	nd	nd
30	G. max™	20	1529±65	3.1±6.9	nd	nd
	A. hypogea™	20	1276±242	<4	nd	nd
	U. europeus™	20	1583±94	<4	nd	nd

* The reciprocal of the antiserum dilution that gave on ELISA reading of 0.5 after 45 min at 37C. Each value represents the mean of 5 mice ±1 standard deviation.

™ Each lectin was substituted with 4 DNP groups

H III

H III
↓

Ile Ser Met Lys ↓

5

_____ LTB coding sequence ATC AGT ATG AAA GCTT
 121 122

Size = 573 bp

10 This fragment was ligated into the vector pUC13 (Messing, 1983) after Hind III digestion and phosphatase treatment of the plasmid using standard conditions. This served to place the remaining polylinker region of pUC13, including a PstI, SalI, XbaI, BamHI, SmaI, SstI and EcoRI sites downstream of the LTB sequence containing DNA insert.

15 2. Creation of Synthetic LHRH Coding Oligonucleotides

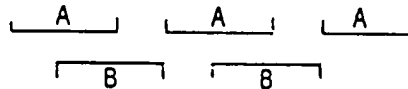
Two oligonucleotides, of 30 bases in length, with sequences described in A and B below, were designed to form overlapping hybrid duplexes, as shown in C, which result in a duplex which will encode linear end to end repeats of the 10 amino acids encoding the peptide hormone LHRH (Schally and Coy, 1983). Role of Peptides and Proteins in Control of Reproduction, McCann and Dhindsa eds, Elsevier Science Publishing Co, Inc. pp 89-110). In this sequence, glutamic acid replaces the normal N-terminal pyroglutamic acid.

A. 5' GAG CAC TGG TCC TAC GGC CTT CGA CCC GGG 3'

B. 5' GTA GGA CCA GTG CTC CCC GGG TCG AAG GCC 3'

25

C.



30

etc.

The oligonucleotides were annealed together for 1 hr at 40 °C in 50 mM NaCl, 10 mM Tris pH 7.5, and filled with Klenow, and then the mixture was ligated into SmaI cut M13 mp18, using standard procedures. M13 phage containing inserts were isolated, and the DNA sequences of the inserts were determined by the dideoxy technique. One recombinant, designated as P29, was chosen for the fused construct. Its DNA sequence, together with the amino acids it encodes, in the region of the insert at the SmaI site is given below.

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TABLE 3.3

Carrier potential of 987P for various antigens

5	Immunogen	Molar Dose	Ratio	(μg)	Immune Response*			
					Antigen		Carrier	
					Serum IgG	Int IgA	Serum IgG	Int IgA
10	987P	-	20	<4	<4	891±76	27.8±13.6	
	987P	-	100	<4	<4	1024±83	84±26.7	
	BSA-987P	1:4	20	<4	<4	84±2.6	<4	
15	BSA-987P	1:5	500	<4	<4	256±49	<4	
	BSA-987P	1:10	500	29.8±7.4	4.6±2.2	675±110	<4	
	BSA-987P	1:20	500	306±88	122±47.6	4263±408	194±38.6	
	BSA-987P	1:40	500	124±47	110±38.4	4705±521	156±55	
20	Flag-987P	1:5	20	1552±361	<4	-	-	
	LPS-987P	1:1**	20	<4	<4	194±28.6	<4	
	PS-987P	1:1**	20	<4	<4	337±96	5.2±6.8	
25	BSA	-	20	<4	<4	-	-	
	Flagellae	-	20	<4	<4	-	-	
	LPS	-	20	12.1±2.7	<4	-	-	
30	PS	-	20	<4	4.2±0.4	-	-	

* See Table 3.1

** Ratio based on weight.

TABLE 3.4

40	Effects of altering dosage of substituted carrier on the immune response			
	Antigen	Dose (μg)	Immune Response (Serum IgG)*	
			Anti-BSA	Anti-Carrier
45	BSAO.03.K99	70	13.9±4.4	776±148
	BSAO.03.K99	140	36.7±14.6	1782±174
	BSAO.03.K99	280	73.5±22.1	1989±215
	BSAO.05.987P	70	36±2.6	891±109
	BSAO.05.987P	140	168±23.7	1552±176
50	BSAO.05.987P	280	337±43.7	2042±180

* See Table 2.1

4. Construction of expression plasmid K66 and expression strain BTA1185

A 573 bp Eco RI fragment of the pUC13 LTB-LHRH fusion plasmid described in 3, which contains the LTB-LHRH fusion coding region in its entirety, was isolated from an agarose gel and ligated into Eco RI cut, phosphatased expression vector pKK223-3 (from Pharmacia). The resultant expression plasmid PBTA K66, placed the expression of the fusion protein under the control of the tac promoter, where expression is induced with IPTG. The plasmid was transformed into *E. coli* host strain JM101 (SupE, thi, (lac-pro AB) [F' traD36, pro AB lacI^q Z M 15]) to give the host vector expression system BTA1185.

5. Production and Purification of LTB/LHRH Fusion Protein for Animal Trials.

The LTB(LHRH)_{3.5} producing strain was grown as described previously. After induction with IPTG for 2 h bacteria were pelleted by centrifugation (3,000 x g, 10 min at 4 °C). Bacteria were then resuspended in dH₂O and lysed in a French Press. After removal of the bacterial debris by centrifugation (18,000 x g, 10 min, at 4 °C) the supernatant was loaded onto an agthio-galactose column (Sigma). The fusion protein was then eluted with 0.5M galactose and dialysed against 0.1M carb/bicarb buffer pH 9.5.

Antigen administration and measurement of the immune response

All oral presentation procedures, antibody collections and ELISA determinations were as described previously.

RESULTS

25 Demonstration of the carrier potential of the mucosal immunogens

All of the mucosal immunogens tested showed the capacity to effectively transport the covalently attached hapten DNP across the intestinal mucosa and to elicit a serum anti-DNP Ab response after feeding of the dinitrophenylated-MI. DNP-modified BSA, however was completely ineffective in eliciting an anti-DNP or anti-BSA response when fed at the concentrations tested (Table 3.1). Initial experiments in which K99 and 987P were complexed to much larger molecules than DNP were unsuccessful in generating immune responses to either the mucosal immunogen or to the molecule coupled to it (Tables 3.2 & 3.2), possibly due to steric interference in the binding of the pili to the mucosal epithelium. It was therefore decided to vary the ratio of antigen to MI. When various ratios of BSA:pili were tested, it was found that when ratios of greater than 1:20 BSA:pili were fed it was not possible to generate either anti-BSA or anti-pili responses even with a dose of 500 ug, demonstrating that it was not possible for the complexes to effectively associate with their mucosal epithelium and to therefore generate an immune response. However when ratios of 1:20 or 1:40 were employed good responses to both BSA and to pili were observed (Tables 3.2 & 3.3). The magnitude of the immune response was readily varied by altering the doses of complex fed (Table 3.4).

Oral administration of LHRH coupled to LTB lead to a significant reduction in the combined uterine and ovarian weights of female mice receiving either 20 or 50 ug LHRH-LTB (P 0.05) (Table 3.5,3.6). No such weight loss was seen with either LHRH or LTB fed alone or together or to intramuscular injection of LHRH-B-galactosidase, LHRH-LTB, or free LHRH. The effect of the weight loss was also seen developmentally as there was a complete absence of mature follicles in the ovaries, thus, the animals were effectively "castrated". There was a slight reduction in reproductive tract weights when mice were fed the genetically constructed LTB-(LHRH)_{3.5} fusion protein (Table 3.6) but in this experiment, the reduction was not significant at the doses tried.

50 EXAMPLE 4

Induction of cell-mediated immunity after oral administration of antigen

Feeding of mucosal immunogens was shown to be effective in eliciting humoral responses as measured by the production of serum and intestinal antibodies. It was not known, however whether there was a concomitant stimulation of a cell mediated immune (CMI) response to the mucosal immunogens.

The following study was designed to compare the CMI generated by oral presentation of a mucosal immunogen with that generated by classic subcutaneous (s.c.) injection of antigen in Complete Freund's

- BEFUS, A.D., and J. Bienenstock. 1982. Factors involved in symbiosis and host resistance at the mucosa-parasite interface. *Prog. Allergy*. 31 : 76.
- BIENENSTOCK, J., and A.D. Befus. 1980. Mucosal Immunology. *Immunology*. 41 : 249.
- BLAKE, M.S., and E.C. Gotschlich. 1984. Purification and partial characterization of the opacity-associated proteins of *Neisseria gonorrhoeae*. 159 : 452.
- 5 BLAND, P.W. & Britton, D.C. (1984) Morphological study of antigen-sampling structures in the rat large intestine. *Infect. Immun.*, 43, 693-699.
- CLARK, S., A. Cahill, C. Stirzaker, P. Greenwood and R. Gregson. 1985. Prevention by vaccination-animal bacteria, p. 481-487. In S. Tzipori (ed.) *Infectious diarrhoea in the young*. Elsevier Science Publishers B.V.
- 10 CONTEY, J.R., and L.R. Inman. 1981. Diarrhea due to *Escherichia coli* strain RDEC-1 in the rabbit. The Peyer's patch as the initial site of attachment and colonization. *J. Infect. Dis.* 43 : 440.
- DALLAS et. al. (1979) Cistrons encoding *Escherichia coli* heat labile toxin *J. Bact.* 139 850-858.
- ELSON, C.O. & EALDING, W. (1984a) Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.*, 132, 2736-2741.
- 15 ELSON, C.O. & EALDING, W. (1984b) Cholera toxin did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated antigen. *J. Immunol.* 133, 2892-2897.
- EVANS, D.G. & Evans, D.J. (1978) New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. *Inf. Immun.*, 21, 638-647.
- De GRAAF, F.K., P. Klemm, and W. Gastra. 1981. Purification, characterization and partial covalent structure of the adhesive antigen K99 of *Escherichia coli*. *Infect. Immun.* 33 : 877.
- 20 De GRAAF, F.K. & Roorda, I. (1982) Production, purification, and characterization of the fimbrial adhesive antigen F41 isolated from calf enteropathogenic *Escherichia coli* strain B41M. *Infect. Immun.*, 36, 751-758.
- FUJITA, K. & Finkelstein, R.A. 1972 Antitoxic immunity in experimental Cholera. *J. Infect. Dis.*, 125, 647-655.
- 25 FUSCO, P., A. To, S. To, and C. Brinton, Jr. 1978. The purification and characterisation of four types of *E. Coli* pili and the specificity of *E. coli* pili for immunity colonization and adhesion, p. 60-70. In C. Miller (ed.), XIIIth U.S. Japan Conference on Cholera, Atlanta, Ga., 1977. National institution of Health, Bethesda, Md.
- GAASTRA, W., and F.K. de Graaf. 1982. Host specific fimbrial adhesion of noninvasive enterotoxigenic *Escherichia coli* strains. *Microbiol. Rev.* 46 : 129.
- 30 GAASTRA, R.A. (1975) An attempt to identify the intestinal receptor for the K88 adhesin by means of a haemagglutination inhibition test using glycoproteins and fractions and sow colostrum. *J. Gen. Microbiol.*, 86, 228-240.
- GIBBONS, R.A. (1975) An attempt to identify the intestinal receptor for the K88 adhesin by means of a haemagglutination inhibition test using glycoproteins and fractions from sow colostrum. *J. Gen. Microbiol.*, 86, 228-240.
- 35 HANSON, D.G., VAZ, N.M., MAIA, L.C.S. & LYNCH, J.M., (1979) Inhibition of specific immune responses by feeding protein antigens. *J. Immunol.*, 123, 2337-2343.
- HERBERT, D., P.J. Phipps, and R.E. Strange. 1985. Carbohydrate analyses, determination of total carbohydrate, p. 265-279. In J.R. Norris, and D.W. Ribben (ed.), *Methods in Microbiology*. v. 5B.
- 40 ISAACSON, R.E., J. Colmenero, and P. Richter. 1981. *Escherichia coli* K99 pili are composed of one subunit species. *FEMS Microbiol. Lett.* 12 : 229.
- ISAACSON, R.E., and P. Richter. 1981. *Escherichia coli* 987P Pilus. Purification and partial characterization. *J. Bacteriol.* 146 : 784.
- KLIPSTEIN, F.A., ENGERT, R.F., & SHORT, H.B. (1980) Protective effect of immunization with heat -labile enterotoxin in gnotobiotic rats monocontaminated with enterotoxigenic *Escherichia coli*. *Inf. Immunol.*, 28, 163-170.
- 45 LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227 : 680.
- LEONG et. al. (1985) Nucleotide sequence comparison between heat labile toxin B-subunit cistrons from *Escherichia coli* of human and porcine origin *Infection and Immunity* 48 73-77.
- 50 LEVINE, M.N., Rennels, M.B., Daya, V. & Hughes, T.P. (1980) Haemagglutination and colonization factors in enterotoxigenic and enteropathogenic *Escherichia coli* that cause diarrhea. *J. Inf. Dis.*, 141, 733-737.
- LITTLE, J.R. & Eisen, H.N. (1967) Preparation of immunogenic 2,4-dinitrophenyl and 2,4,6- trinitrophenyl proteins. In "Methods in Immunology and Immunochemistry" (Ed. Williams, C.A. & Chase, M.W.). I, p.128.
- 55 Academic Press, New York.
- MESSIER, B., and C.P. Le Blond (1960). Cell proliferation and migration as revealed by radioautography after injection of thymidine -H3 into male rats and mice. *An. J. Anat.* 106 : 247.
- MESSING (1983) New M13 Vectors for cloning *Methods in Enzymology* 101 20-78

5. A complex according to claim 4, wherein the allergen is derived from grass pollen, weed pollen, tree pollen, plant pollen, cat hair, dog hair, pig hair or other epithelia, or house dust, wheat chaff or kapok.
6. A complex according to claim 1 or 2, wherein the immunogen is a surface protein derived from an influenza, measles, Rubella, smallpox, yellow fever, diphtheria, tetanus, cholera, plague, Typhus or BCG causing agent, Haemophilus influenza, Neisseria catarrhalis, Klebsiella pneumoniae, a pneumococcus or a Streptococcus; or a pilus derived from E. coli, N. gonorrhoeae, N. meningitidis, N. catarrhalis, a Yersinia species, Pseudomonas aeruginosa, a Pseudomonas species, Moraxella bovis, Bacteroides nodosus, a Staphylococci species, a Streptococci species or a Bordetella species.
7. A complex according to claim 1 or 2, wherein the immunogen is a surface polysaccharide derived from a diphtheria, tetanus, cholera, plague, Typhus or BCG causing agent, Haemophilus influenza, Neisseria catarrhalis, Klebsiella pneumoniae, a pneumococcus or a Streptococcus.
8. A complex according to claim 1 or 2, wherein the immunogen is a secretory product derived from a diphtheria, tetanus, cholera, plague, Typhus or BCG causing agent, Haemophilus influenza, Neisseria catarrhalis, Klebsiella pneumoniae, a Pneumococcus or a Streptococcus.
9. A complex according to any one of claims 6 to 8, wherein the surface protein, surface polysaccharide or secretory product is derived from Streptococcus mutans.
10. A complex according to claim 1, wherein the carrier molecule is the heat labile toxin of enterotoxigenic E. coli.
11. A complex according to claim 1, wherein the bacterial adhesin is the K99 or 987P pilus of E. coli.
12. A complex according to claim 1, wherein the bacterial adhesin is CFAI, CFAll, K88 or F41.
13. A complex according to claim 1, wherein the lectin is Concanavalin A, Pokeweed mitogen or the lectin from Lens culinaris, Helix pomatia, Glycine max, Arachis hypogaea, or Ulex europeus.
14. A complex according to claim 1, wherein the lectin is Abrin, Asparagus pea, Broad bean, Camel's foot tree, Caster bean, Fava bean, Green marine algae, Hairy vetch, Horsegum, Horseshoe crab, Jack bean, Japanese wisteria, Jequirity bean, Scotch laburnum, Lima bean, Limulin, Lotus, European mistletoe, Mung bean, Osage orange, Pagoda tree, Garden pea, Potato, Red Kidney bean, Red marine algae, Siberian pea tree, edible snail, garden snail, Spindle tree, Sweet pea, Tomato, Wheat germ or Winged pea lectin.
15. A complex according to claim 1, wherein the viral haemagglutinin is a haemagglutinin derived from influenza, measles, Rubella, smallpox or yellow fever virus.
16. A complex according to any one of claims 1 to 3, wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof, of luteinizing hormone releasing hormone and the carrier molecule is all, part, an analogue, homologue, derivative or combination thereof, of LTB.
17. A process for the production of a complex according to any one of claims 1 to 16, which process comprises:
 - (a) reacting the immunogen with the carrier molecule to form said complex;
 - (b) chemically modifying the immunogen to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;
 - (c) chemically modifying the carrier molecule to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;
 - (d) chemically modifying the immunogen and the carrier molecule to provide functional groups capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;
 - (e) reacting the immunogen with at least one linking agent, and reacting the immunogen and carrier molecule to form said complex;

homologue, derivative or combination thereof, of a bacterial adhesin, a viral haemagglutinin, a toxin, or a binding subunit of a toxin, or a lectin, but not including a DNA molecule coding for an immunogen carrier complex wherein the immunogen is naturally linked to the carrier molecule.

- 5 30. A recombinant DNA molecule according to claim 29 also comprising vector DNA.
31. A recombinant DNA molecule according to claim 30, wherein the vector DNA is selected from plasmid, viral, bacteriophage, or cosmid DNA.
- 10 32. A recombinant DNA molecule according to claim 29, wherein the DNA sequence acts as a coding sequence for an antigen or hapten fused to the amino acid sequence of the carrier molecule.
33. A recombinant DNA molecule according to claim 29, wherein the DNA sequence acts as a coding
15 sequence for all, part, an analogue, homologue, derivative of LHRH fused to the amino acid sequence of the carrier molecule.
34. A medicament which comprises an immunogen suitable for vaccination purposes linked to a carrier molecule capable of specifically binding to the epithelial mucosa of a vertebrate host, wherein both the
20 immunological activity of the immunogen and the capacity of the carrier to bind specifically to the mucosal epithelium is substantially maintained, and the carrier molecule is an epithelial mucosal binding molecule selected from all, a part, an analogue, a homologue, a derivative or combination thereof, of a bacterial adhesin, viral haemagglutinin, a toxin, or a binding subunit of a toxin, or a lectin, together with a pharmaceutically acceptable carrier or diluent but not including immunogen-carrier complexes wherein the immunogen is naturally linked to the carrier molecule.
- 25 35. A medicament according to claim 34, adapted for oral administration.
36. A medicament according to claim 34, adapted for nasal administration.
- 30 37. A medicament according to any one of claims 34 to 36, wherein said medicament is in capsule, tablet, slow release, elixir, gel, paste or nasal spray form.
38. A medicament according to any one of claims 34 to 37, which additionally comprises a dietary
35 molecule to selectively modulate the magnitude and/or type of immune response to the immunogen of the medicament.
39. A medicament according to claim 38, wherein the dietary molecule is selected from amino acids, vitamins, monosaccharides and oligosaccharides.
- 40 40. A medicament according to claim 38 or claim 39, wherein the dietary molecule is selected from vitamin A, vitamin B₁, vitamin B₂, vitamin B₆, vitamin B₁₂, vitamin C, vitamin D, vitamin E, fructose, lactose, mannose, melibiose, sorbitol or xylose.

Patentansprüche

- 45 1. Komplex,
dadurch gekennzeichnet,
daß er ein für Impfzwecke geeignetes Immunogen umfaßt, das an ein Trägermolekül gebunden ist, welches zur spezifischen Bindung an die epitheliale Schleimhaut eines Wirbeltierwirts geeignet ist,
50 worin sowohl die immunologische Aktivität des Immunogens als auch die Eignung des Trägers, spezifisch an das Schleimhautepithel zu binden, im wesentlichen erhalten bleibt, und das Trägermolekül ein zur Bindung an das Schleimhautepithel geeignetes Molekül ist, ausgewählt aus dem Ganzen, einem Teil, einem Analogon, einem Homologen, einem Derivat oder einer Kombination hiervon eines bakteriellen Adhesins, eines viralen Hämagglutinins, eines Toxins oder einer Bindungsuntereinheit eines
55 Toxins oder eines Lectins, wobei jedoch Immunogen-Trägerkomplexe ausgenommen sind, worin das Immunogen natürlicherweise an das Trägermolekül gebunden ist.

eine Kombination hiervon von LTB ist.

17. Verfahren zur Herstellung eines Komplexes nach einem der Ansprüche 1 bis 16, wobei das Verfahren die folgenden Schritte aufweist:
 - 5 (a) Umsetzung des Immunogens mit dem Trägermolekül, um den Komplex zu bilden;
 - (b) chemische Modifikation des Immunogens, um wenigstens eine funktionelle Gruppe bereitzustellen, die in der Lage ist, eine chemische Bindung auszubilden, und Umsetzung des Immunogens und des Trägermoleküls, um den Komplex zu bilden;
 - (c) chemische Modifikation des Trägermoleküls, um wenigstens eine funktionelle Gruppe bereitzustellen, die in der Lage ist, eine chemische Bindung auszubilden, und Umsetzung des Immunogens und des Trägermoleküls, um den Komplex auszubilden;
 - 10 (d) chemische Modifikation des Immunogens und des Trägermoleküls, um funktionelle Gruppen bereitzustellen, die in der Lage sind, eine chemische Bindung auszubilden, und Umsetzung des Immunogens und des Trägermoleküls, um den Komplex auszubilden;
 - 15 (e) Umsetzung des Immunogens mit wenigstens einem Bindungsmittel, und Umsetzung des Immunogens und des Trägermoleküls, um den Komplex auszubilden;
 - (f) Umsetzung des Trägermoleküls mit wenigstens einem Bindungsmittel, und Umsetzung des Immunogens und des Trägermoleküls, um den Komplex auszubilden;
 - (g) Umsetzung des Immunogens und des Trägermoleküls mit wenigstens einem Bindungsmittel, und Umsetzung des Immunogens und des Trägermoleküls, um den Komplex auszubilden; oder
 - 20 (h) eine Kombination aus einem der oben genannten Verfahrensschritte.
18. Verfahren zur Herstellung eines Komplexes nach einem der Ansprüche 1 bis 3, wobei das Verfahren die nachfolgenden Verfahrensschritte aufweist:
 - 25 Bereitstellen eines rekombinanten DNA-Moleküls, das eine erste DNA-Sequenz aufweist, die bei Expression für die Aminosäuresequenz des Immunogens codiert, eine zweite DNA-Sequenz, die bei Expression für die Aminosäuresequenz des Trägermoleküls codiert und eine Vektor-DNA; Transformation eines Wirts mit dem rekombinanten DNA-Molekül, so daß der Wirt in der Lage ist, ein Hybrid-Proteinprodukt zu exprimieren, das den Komplex umfaßt; Kultivierung des Wirts, um die Expression zu erhalten und Sammeln des Hybrid-Proteinprodukts.
 - 30
19. Verfahren zur Herstellung eines Komplexes nach einem der Ansprüche 1 bis 16, wobei das Verfahren die nachfolgenden Verfahrensschritte aufweist:
 - 35 (a) chemische Synthese des Immunogens und/oder des Trägermoleküls und Ausbildung des Komplexes durch Reaktionen nach dem Verfahren des Anspruchs 17; oder
 - (b) Synthese eines Hybridpeptids, das Aminosäuresequenzen des Immunogens und des Trägermoleküls aufweist.
20. Verfahren nach Anspruch 19, worin das Peptid durch Festphasen-, enzymatische oder manuelle Peptidsynthese hergestellt wird.
 - 40
21. Verfahren nach Anspruch 20, worin das synthetisierte Immunogen oder Trägermolekül an das Trägermolekül oder Immunogen gekoppelt ist, während es an ein Harz eines Festphasen-Peptidsyntheseapparats gebunden ist.
 - 45
22. Verfahren nach einem der Ansprüche 19 bis 21, worin das Immunogen das Ganze, ein Teil, ein Analogon, ein Homologes, ein Derivat oder eine Kombination hiervon des Luteinisierungshormon-freisetzenden Hormons (LHRH) ist.
23. Verfahren nach Anspruch 17, worin das Trägermolekül LTB und das Verbindungsmittel Isoglutaraldehyd ist.
 - 50
24. Komplex nach einem der Ansprüche 1 bis 3, hergestellt durch ein Verfahren nach einem der Ansprüche 17 bis 21.
 - 55
25. Expressionsprodukt eines transformierten Wirts, dessen Produkt einen Komplex nach einem der Ansprüche 1 bis 3 umfaßt.

38. Arzneimittel nach einem der Ansprüche 34 bis 37, das zusätzlich ein Nahrungsmolekül aufweist, um die Größenordnung und/oder die Art der Immunantwort auf das Immunogen des Arzneimittels selektiv zu modulieren.
- 5 39. Arzneimittel nach Anspruch 38, wobei das Nahrungsmolekül aus Aminosäuren, Vitaminen, Monosacchariden und Oligosacchariden ausgewählt wird.
40. Arzneimittel nach Anspruch 38 oder Anspruch 39, worin das Nahrungsmolekül ausgewählt wird aus Vitamin A, Vitamin B₁, Vitamin B₂, Vitamin B₆, Vitamin B₁₂, Vitamin C, Vitamin D, Vitamin E, Fructose, Lactose, Mannose, Melibiose, Sorbitol oder Xylose.
- 10

Revendications

1. Complexe caractérisé en ce qu'il comprend un immunogène vaccinal lié à une molécule porteuse capable de se fixer de manière spécifique à la muqueuse épithéliale d'un hôte vertébré, dans lequel l'activité immunologique de l'immunogène et la capacité du porteur à se fixer de manière spécifique à la muqueuse épithéliale sont toutes deux essentiellement conservées, la molécule porteuse étant une molécule capable de se fixer à la muqueuse épithéliale et choisie parmi la molécule entière, une partie, un analogue, un homologue, un dérivé ou une combinaison de ces derniers, d'une adhésine bactérienne, d'une hémagglutinine virale, d'une toxine, ou d'une sous-unité de fixation de toxine ou de lectine; complexe ne comprenant pas les complexes immunogène-porteur dans lesquels l'immunogène est lié à la molécule porteuse à l'état naturel.
- 15
2. Complexe selon la revendication 1, dans lequel ledit immunogène est choisi parmi un antigène ou un haptène, ou la molécule entière, une partie ou plusieurs, un analogue, un homologue, un dérivé ou une combinaison de ces derniers, d'une hormone, ou d'un agent thérapeutique.
- 25
3. Complexe selon la revendication 2, dans lequel l'immunogène est constitué de la molécule entière, d'une partie, d'un analogue, d'un homologue, d'un dérivé ou d'une combinaison de ces derniers, d'une hormone choisie parmi FSH, HGH, LHRH ou l'inhibine.
- 30
4. Complexe selon la revendication 1 ou 2, dans lequel l'immunogène est un allergène.
5. Complexe selon la revendication 4, dans lequel l'allergène est dérivé du pollen des graminées, du pollen des mauvaises herbes, du pollen des arbres, du pollen des plantes, du poil de chat, du poil de chien, du poil de porc ou autres tissus épithéliaux, ou la poussière ménagère, la balle de blé ou le kapokier.
- 35
6. Complexe selon la revendication 1 ou 2, dans lequel l'immunogène est une protéine de surface dérivée de l'agent étiologique de la grippe, de la rougeole, de la rubéole, de la variole, de la fièvre jaune, de la diphtérie, du tétanos, du choléra, de la peste, du typhus, ou du BCG, Haemophilus influenza, Neisseria catarrhalis, Klebsiella pneumoniae, un pneumocoque, ou un Streptocoque; ou une fimbriae dérivée d'E. coli, N. gonorrhea, N. méningitidis, N. catarrhalis, une espèce de Yersinia, Pseudomonas aeruginosa, une espèce de Pseudomonas, Moraxella bovis, Bacteroides nodosus, une espèce de Staphylocoques, une espèce de Streptocoques ou une espèce de Bordetella.
- 40
7. Complexe selon la revendication 1 ou 2, dans lequel l'immunogène est un polysaccharide de surface dérivé de l'agent étiologique de la diphtérie, du tétanos, du choléra, de la peste, du typhus, ou du BCG, Haemophilus influenza, Neisseria catarrhalis, Klebsiella pneumoniae, un pneumocoque, ou un Streptocoque.
- 50
8. Complexe selon la revendication 1 ou 2, dans lequel l'immunogène est un produit sécrétoire dérivé de l'agent étiologique de la diphtérie, du tétanos, du choléra, de la peste, du typhus ou du BCG, Haemophilus influenza, Neisseria catarrhalis, Klebsiella pneumoniae, un pneumocoque, ou un Streptocoque.
- 55
9. Complexe selon l'une quelconque des revendications 6 à 8 dans lequel la protéine de surface, le polysaccharide de surface ou le produit sécrétoire sont dérivés de Streptococcus mutans.

19. Procédé pour la production d'un complexe selon l'une quelconque des revendications 1 à 16, lequel procédé consistant à
 - (a) synthétiser par voie chimique l'immunogène et/ou la molécule porteuse, et à générer le complexe au moyen des réactions du procédé selon la revendication 17; ou à
 - (b) synthétiser un peptide hybride comportant la séquence d'acides aminés de l'immunogène et celle de la molécule porteuse.
20. Procédé selon la revendication 19, dans lequel on prépare le peptide par synthèse peptidique enzymatique, manuelle ou sur phase solide.
21. Procédé selon la revendication 20, dans lequel on couple l'immunogène synthétisé ou la molécule porteuse après fixation sur la résine de la phase solide du synthétiseur peptidique à la molécule porteuse ou à l'immunogène respectivement.
22. Procédé selon l'une quelconque des revendications 19 à 21, dans lequel l'immunogène est la molécule entière, une partie, un analogue, un homologue, un dérivé ou une combinaison de ces derniers, de la lutéostimuline (LHRH).
23. Procédé selon la revendication 17, dans lequel la molécule porteuse est LTB et l'agent réticulant est l'isoglutaraldéhyde.
24. Complexe selon l'une quelconque des revendications 1 à 3, fabriqué par un procédé selon l'une quelconque des revendications 17 à 21.
25. Produit d'expression d'un hôte transformé; lequel produit comprenant un complexe selon l'une quelconque des revendications 1 à 3.
26. Hôte transformé avec une molécule d'ADN recombinant comportant une première séquence d'ADN qui lors de son expression code pour les séquences d'acides aminés d'un immunogène vaccinal et une seconde séquence d'ADN qui lors de son expression code pour la séquence d'acides aminés d'une molécule porteuse capable de se lier à la muqueuse épithéliale, laquelle molécule porteuse étant une molécule capable de se lier à la muqueuse épithéliale et choisie parmi la molécule entière, une partie, un analogue, un homologue, un dérivé ou une combinaison de ces derniers, d'une adhésine bactérienne, d'une hémagglutinine virale, d'une toxine, ou d'une sous-unité de fixation d'une toxine, ou d'une lectine, hôte dans lequel l'immunogène et la molécule porteuse génèrent un polypeptide de fusion; molécule ne comprenant pas une molécule d'ADN codant pour un complexe immunogène-porteur dans lequel l'immunogène est lié à la molécule porteuse à l'état naturel.
27. Hôte transformé selon la revendication 26, dans lequel l'hôte est une bactérie Gram négative, Gram positive, une levure, une moisissure, ou une cellule eucaryote supérieure.
28. Hôte transformé selon la revendication 26 ou la revendication 27, dans lequel l'hôte est E. coli.
29. Molécule d'ADN recombinant comprenant une séquence d'ADN qui lors de son expression code pour un polypeptide de fusion qui comporte la séquence d'acides aminés d'un immunogène vaccinal, et la séquence d'acides aminés d'une molécule porteuse capable de se lier à la muqueuse épithéliale, laquelle molécule porteuse étant une molécule capable de se lier à la muqueuse épithéliale et choisie parmi la molécule entière, une partie, un analogue, un homologue, un dérivé ou une combinaison de ces derniers, d'une adhésine bactérienne, d'une hémagglutinine virale, d'une toxine, ou d'une sous-unité de liaison d'une toxine, ou d'une lectine, molécule ne comprenant pas une molécule d'ADN codant pour un complexe immunogène-porteur dans lequel l'immunogène est lié à la molécule porteuse à l'état naturel.
30. Molécule d'ADN recombinant selon la revendication 29 comprenant en outre un vecteur d'ADN.
31. Molécule d'ADN recombinant selon la revendication 30, dans laquelle le vecteur d'ADN est choisi parmi un ADN plasmidique, viral, de bactériophage ou de cosmide.

	1	5	10
Type 1	Ala-Ala-Thr-Thr-Val-Asn-Gly-Gly-Thr-Val-His-Phe-Lys-Gly-		
K88	Trp-Met-Thr-Gly-Asp-Phe-Asn-Gly-Ser-Val-Asp-Ile-Gly-Gly-		
K99	Asn-Thr-Gly-Thr-Ile-Asn-Phe-Asn-Gly-Lys-Ile-Thr-Ser-Ala-		
987P	Ala-Pro-Val-Glu-Asn-Asn-Thr-Cys-Gln-Ala-Asn-Leu-Asp-Phe-		
Neisseria	Phe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Ile-Ala-Ile-Val-Gly-		
	15	20	25
Type 1	Glu-Val-Val-Asn-Ala-Ala-		
K88	Ser-Ile-Thr-Ala-Asp-Asp-Tyr-Arg-		
K99	Thr-Cys-Thr-Ile-Glu-Pro-Glu-Ala-		
987P	Thr-Gly-Lys-Val-Thr-Ala- x -Leu-		
Neisseria	Ile-Leu-Ala-Ala-Val-Ala-Leu-Pro-		

Fig.1 N-terminal amino acid sequence of the 987P pilin subunit. The N-terminal sequences of other pilin proteins are given for comparison.

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(54) Title: TREATMENT OF <i>H. PYLORI</i> ASSOCIATED GASTRODUODENAL DISEASE (57) Abstract A method for the treatment of <i>Helicobacter</i> infection, preferably <i>H. pylori</i> infection in a mammalian host such as a human comprises administration to the infected host of an immunologically effective amount of one or more <i>Helicobacter</i> antigen(s), preferably in association with a mucosal adjuvant such as cholera toxin or <i>E.coli</i> heat labile toxin. <i>ℒ</i>		

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TREATMENT OF *H. PYLORI* ASSOCIATED GASTRODUODENAL DISEASE

5

FIELD OF THE INVENTION

This invention relates to the treatment of gastroduodenal disease associated with *Helicobacter pylori* infection and in particular it relates to the use of active immunisation as a treatment for *H. pylori* -associated gastroduodenal

10 disease.

BACKGROUND OF THE INVENTION

The bacterium, *Helicobacter pylori*, is now well established as a major gastroduodenal pathogen, and more than 50% of the world population is infected

15 with this organism which causes gastritis of varying severity. While no symptoms are apparent in a great proportion of infected persons, in a significant number of *H. pylori* infected persons overt disease may result. The majority (95%) of duodenal ulcers are associated with *H. pylori* infection; a causal role is shown by treatment studies which indicate that if the organisms can be eradicated at the

20 time of ulcer healing then the ulcers do not recur - in contrast to 80% recurrence rate at one year in those who remain infected with the organisms. Furthermore, up to 80% of gastric ulcers are thought to be *H. pylori* associated (Blaser, 1992).

There is now increasing evidence of the harmful consequence of long term

25 *H. pylori* infection. In countries such as China, Colombia and Japan the bacterium is picked up very early in life, and in these persons the gastritis slowly progresses until after 30-40 years of continual infection, severe gastric atrophy appears. Gastric atrophy is well documented as being the precursor lesion for gastric cancer, although the actual cancer that develops in an atrophied stomach

30 is dependent on a myriad of other factors including diet. However, all the evidence to date would suggest that the cancer would not develop if it was

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possible to remove the *H. pylori* infection at an early age before the atrophy had developed (Parsonnet *et al.* , 1991).

There is no laboratory animal model of *H. pylori* infection that can be used
5 for large scale assessment of new anti-*H. pylori* therapies. However, a
Helicobacter felis mouse model of gastric *Helicobacter* infection has been
developed that has proved extremely useful in the screening of the potential of
new antimicrobial therapeutic regimens. *H. felis* is a spiral shaped bacterium
that is very closely related to *H. pylori* . This bacterium colonises the stomach
10 of mice in a very similar way to *H. pylori* in the human, i.e. the main ecological
niche is gastric mucus and the localisation of colonisation is antral dominant. In
germfree mice, *H. felis* infection induces a gastritis that is very similar to the
human *H. pylori* infection with a chronic inflammation accompanied by
polymorphonuclear leucocyte infiltration. Infection with each organism results in
15 the induction of a similar raised immune response against *H. pylori* and *H. felis*
respectively (Lee *et al.* , 1990).

The *H. felis* mouse model has proved to be very predictive of the efficacy
of anti-*H. pylori* agents in humans. Thus, monotherapy with agents with high *in*
20 *vitro* activity such as erythromycin show no significant *in vivo* effect against *H.*
felis in mice, just as erythromycin has no anti-*H. pylori* effect in humans despite
high antimicrobial effects *in vitro*. In contrast, the triple therapy regimens of a
bismuth compound, metronidazole, and tetracycline or amoxycillin lead to a very
high eradication rate in *H. felis* infected mice (Dick-Hegedus and Lee, 1991).
25 Such triple therapies are the most successful human anti-*H. pylori* regimens, and
at the present time are recommended as the first choice for anti-*H. pylori*
therapy. However, established *Helicobacter* infections are difficult to treat, and
current chemotherapeutic regimens remain suboptimal due to problems with
efficacy, toxicity, drug resistance and reinfection (O'Connor, 1992).

30

Active immunisation of already infected patients has not been proven
efficacious for any clinically manifest human infectious disease (Burke, 1992).

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Given that *H. pylori* infections persist for long periods, if not the life of the infected individual, despite the presence of a vigorous immune response that includes a high level of circulating IgG antibody in the serum and the demonstration of local specific IgA antibody in the gastric mucosa, it has been
5 considered that active immunisation was unlikely to be effective in therapy (Goodwin, 1993). Indeed, Czinn *et al.* (1993) in proposing that oral vaccination may be a feasible approach for the prevention of *H. pylori* infection in humans (based on an evaluation of an oral immunisation protocol in the *H. felis* mouse model), suggested that once infection is established neither antibody nor
10 antibiotics are very effective at eradication.

Varga *et al.* (1992) have reported that a *H. pylori* vaccine prepared from organisms derived from a patient, and injected parenterally into that patient, resulted in an allergic reaction and failure to eradicate the organism.

15

Surprisingly, it has now been discovered for the first time that there is indeed a therapeutic potential for active immunisation against gastric *Helicobacter* infection. Furthermore, it has been discovered that oral administration of *H. pylori* antigen, with a suitable mucosal adjuvant, does not result in allergic or
20 hypersensitivity symptoms, but results in suppression or eradication of the infecting organisms from the gastric mucosa.

SUMMARY OF THE INVENTION

According to one aspect of the present invention, there is provided a
25 method for the treatment of *Helicobacter* infection in a mammalian host, which comprises the oral administration to said infected host of an immunologically effective amount of one or more *Helicobacter* antigen(s), optionally in association with a mucosal adjuvant.

30 In another aspect, there is provided a vaccine composition for the treatment of *Helicobacter* infection in a mammalian host, which comprises an

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immunologically effective amount of one or more *Helicobacter* antigen(s), optionally in association with a mucosal adjuvant.

In yet another aspect, the present invention provides the use of a vaccine
5 composition comprising an immunologically effective amount of one or more
Helicobacter antigen(s), optionally in association with a mucosal adjuvant, in the
treatment of *Helicobacter* infection in a mammalian host.

Throughout this specification and the claims which follow, unless the
10 context requires otherwise, the word "comprise", or variations such as
"comprises" or "comprising", will be understood to imply the inclusion of a stated
integer or group of integers but not the exclusion of any other integer or group of
integers.

15 By use of the term "immunologically effective amount" herein, it is meant
that the administration of that amount to an individual infected host, either in a
single dose or as part of a series, is effective for treatment of *Helicobacter*
infection. This amount varies depending upon the health and physical condition
of the individual to be treated, the taxonomic group of individual to be treated, the
20 capacity of the individual's immune system to synthesise antibodies, the degree
of protection desired, the formulation of the vaccine, the assessment of the
medical situation, and other relevant factors. It is expected that the amount will
fall in a relatively broad range that can be determined through routine trials.

25 DETAILED DESCRIPTION OF THE INVENTION

The *Helicobacter* antigen(s) used in accordance with the present invention
may be *H. felis* antigen(s), or more preferably *H. pylori* antigen(s). In a
particularly preferred aspect of the present invention, a vaccine composition
comprising *H. pylori* antigen(s) in association with a mucosal adjuvant is used
30 in the treatment of *H. pylori* infection in a human patient.

- 5 -

Preferably, the *Helicobacter* antigen(s) comprise a bacterial sonicate, and in particular a *H. pylori* sonicate. More preferably, the *Helicobacter* antigen(s) used in accordance with the present invention comprise inactivated whole bacterial cells of *H. pylori*.

5

Alternatively, the *Helicobacter* antigen(s) used in accordance with the present invention may comprise one or more individual antigens, particularly one or more *H. pylori* antigens such as *H. pylori* urease, or *H. pylori* cytotoxin (CT), Cytotoxin Associated Immunodominant (CAI) antigen or heat shock protein (hsp) as disclosed by way of example in International Patent Publication No. WO 93/18150.

10

One mucosal adjuvant which is optionally, and preferably, administered with the *Helicobacter* antigen(s) to the infected host is cholera toxin. Another preferred mucosal adjuvant which may be administered with the *Helicobacter* antigen(s) is *E.coli* heat labile toxin (*E.coli* HLT). Mucosal adjuvants other than cholera toxin and *E.coli* HLT which may be used in accordance with the present invention include non-toxic derivatives of cholera toxin, such as the B sub-unit (CTB), chemically modified cholera toxin, or related proteins produced by modification of the cholera toxin amino acid sequence. Each of these molecules with mucosal adjuvant or delivery properties may be added to, or conjugated with, the *Helicobacter* antigen(s). Other compounds with mucosal adjuvant or delivery activity may be used, such as: bile; polycations such as DEAE-dextran and polyornithine; detergents such as sodium dodecyl benzene sulphate; lipid-conjugated materials; antibiotics such as streptomycin; vitamin A; and other compounds that alter the structural or functional integrity of mucosal surfaces. Other mucosally active compounds include derivatives of microbial structures such as MDP; acridine and cimetidine.

15

20

25

Helicobacter antigen(s) may be delivered in accordance with this invention in ISCOMS (immune stimulating complexes), ISCOMS containing CTB, liposomes or encapsulated in compounds such as acrylates or poly(DL-lactide-co-glycoside)

30

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to form microspheres of a size suited to adsorption by M cells. Alternatively, micro or nanoparticles may be covalently attached to molecules such as vitamin B12 which have specific gut receptors. Antigen(s) may also be incorporated into oily emulsions and delivered orally. An extensive though not exhaustive list of
5 adjuvants can be found in Cox and Coulter, 1992.

Other adjuvants, as well as conventional pharmaceutically acceptable carriers, excipients, buffers or diluents, may also be included in the therapeutic vaccine composition of this invention. The vaccine composition may, for
10 example, be formulated in enteric coated gelatine capsules including sodium bicarbonate buffers together with the *Helicobacter* antigen(s) and mucosal adjuvant.

Generally, a vaccine composition in accordance with the present invention
15 will comprise an immunologically effective amount of *Helicobacter* antigen(s), and optionally a mucosal adjuvant, in conjunction with one or more conventional pharmaceutically acceptable carriers and/or diluents. As used herein "pharmaceutically acceptable carriers and/or diluents" include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and
20 antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and is described by way of example in *Remington's Pharmaceutical Sciences*, 18th Edition, 1990, Mack Publishing Company, Pennsylvania, U.S.A..

25 The pharmaceutical composition of this invention may be orally administered directly to the mammalian host, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatine capsule, or it may be compressed into tablets, or it may be incorporated directly with the solid or liquid food of the diet. For oral therapeutic
30 administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The percentage of active component

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in the compositions and preparations may of course be varied and is such that a suitable dosage will be obtained to be immunologically effective.

Solid oral dosage units such as tablets, troches, pills, capsules and the like
5 may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of
10 wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active component,
15 sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

20 The vaccine composition of the invention is administered orally in amounts readily determined by persons of ordinary skill in this art. Thus, for adults a suitable dosage would be in the range of 10 μg to 10 g, for example 50 μg to 3g. Similar dosage ranges would be applicable for children.

25 As noted above, a suitable mucosal adjuvant is cholera toxin. The amount of mucosal adjuvant employed depends on the type of mucosal adjuvant used. For example, when the mucosal adjuvant is cholera toxin, it is suitably used in an amount of 10 nanogram to 50 μg , for example 01 μg to 10 μg . When the mucosal adjuvant is *E.coli* heat labile toxin, suitable amounts are 1 μg to 1 mg,
30 for example 5 μg to 50 μg .

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In work leading to the present invention, active immunisation of mice previously infected with *H. felis*, with oral doses of cholera toxin or *E.coli* HLT adjuvant and a whole cell *H. felis* or *H. pylori* sonicate, result in the clearance of *H. felis* from the gastric mucosa. It is therefore anticipated that active immunisation of infected humans with oral doses of a mucosal adjuvant with *H. pylori* antigen(s) will result in the clearance of *H. pylori* from the gastric mucosa. Based on previous studies with this model using anti-*H. pylori* agents, it is considered that this is the first evidence of the therapeutic potential of active immunisation with *H. pylori* vaccines, and indicates that a vaccine composition for the therapy of human *H. pylori*-associated gastroduodenal disease is a preparation of *Helicobacter* antigen(s), optionally and preferably combined with a mucosal adjuvant.

It will be apparent to persons skilled in the field that effective treatment of *Helicobacter pylori* infection in humans with an oral vaccine composition of *Helicobacter* antigen(s) which will eradicate or suppress the infection will provide a significant therapeutic benefit via the suppression or elimination of gastritis, prevention of peptic ulcer relapse and reduction in the harmful sequelae of *Helicobacter pylori* infection including peptic ulceration and gastric cancer.

The present invention is further illustrated in the following, non-limiting Examples.

EXAMPLE 1

One hundred and sixty female SPF mice from the Animal Breeding Unit of the University of New South Wales, Australia, were infected with four oral doses of 10^9 - 10^{10} living *Helicobacter felis* (ATCC culture 49179) given two days apart.

Bacteria were grown in plastic Petri dishes on Blood Agar Base No.2, 3.8% w/v (Oxoid, Basingstoke, U.K.) with 7% v/v whole horse blood (Oxoid), containing amphotericin B (Fungizone, Squibb, Princeton, N.J., USA) 2.5 mg/l; trimethoprim

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(Sigma, St.Louis, MO, USA), 10 mg/l. Plates were incubated in a microaerophilic humid atmosphere (Oxoid, BR56) at 37°C for 48 hours.

Sonicates were prepared by growth of the organisms, as described above,
5 followed by harvesting of the organisms in 0.1 molar phosphate buffered saline (PBS). The cells were washed, collected by centrifugation, washed once in PBS, and resuspended in fresh PBS. The cells were then sonicated at the rate of one minute per ml of cell suspension (50% duty cycle) using a B-30 Branson Cell Disrupter. The sonicate was stored at -20°C.

10

On days 28, 42, 44 and 47 after administration of the last infecting dose of *H. felis*, 20 of the mice were given orally 0.2 ml of a suspension containing 10 µg of cholera toxin (Sigma C 3012) and a sonicate of *H. felis* containing 1 mg protein (BIO-RAD DC protein assay).

15

Samples of antral mucosa were tested for infection using a rapid microtitre urease test as described previously (Lee *et al.*, 1990). This test has been validated as being highly predictive of *H. felis* gastric infection. Groups of 40 mice (20 vaccinates and 20 controls) were euthanased at intervals of 1 week, 1
20 month, 2 months and 3 months after the last dose of vaccine.

The results are shown in Table 1.

These results show that treatment of *H.felis* infected mice with an oral
25 vaccine comprised of Helicobacter antigens and a mucosal adjuvant, results in cure of the infection in a significant proportion of mice. This effect is evident 1 week after cessation of therapy, and continues for at least 3 months, demonstrating that the mice have been cured of their infection.

- 10 -

TABLE 1

Immunisation	Proportion of <i>H.felis</i> infected mice			
	1 week	1 month	2 months	3 months
Nil	19/19	20/20	18/19	13/19
Sonicate plus CT	2/20	3/20	6/20	1/17
	P<0.0001*	P<0.0001	P<0.05	P<0.0001

* Fisher's exact test (two tailed).

EXAMPLE 2

One hundred female BALB/c mice from the Animal Breeding Unit of the University of New South Wales, Australia, were infected with 3 oral doses of 10^8 living *Helicobacter felis* (ATCC culture 49179) given 2 days apart, i.e. days 1, 3 and 5.

Bacteria were grown in plastic Petri dishes on Blood Agar Base No. 2, 3.8% w/v (Oxoid, Basingstoke, U.K.) with 7% v/v whole horse blood), (Oxoid), containing amphotericin B (Fungizone, Squibb, Princeton, N.J., USA) 2.5 mg/l; trimethoprim (Sigma, St.Louis, MO, USA), 10 mg/l. Plates were incubated in a microaerophilic humid atmosphere (Oxoid, BR56) at 37°C for 48 hours.

Sonicates were prepared by growth of the organisms, as described above, followed by harvesting of the organisms in 0.1 molar phosphate buffered saline (PBS). The cells were washed collected by centrifugation, washed once in PBS, and resuspended in fresh PBS. The cells were then sonicated at the rate of one per minute per ml of cell suspension (50% duty cycle) using a B-30 Branson Cell Disrupter. The sonicate was stored at -20°C.

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On days 21, 35, 37, and 40 after administration of the last infecting dose of *H. felis*, 20 mice were each given orally 0.2 ml of a solution containing 10 ug of cholera toxin (Sigma C 3012), 20 mice were each given orally 0.2 ml of a suspension containing 10 ug of cholera toxin and a sonicate of *H. felis* containing 1 mg protein (BIO-RAD DC protein assay), 20 mice were each given orally 0.2 ml of a suspension containing a sonicate of *H. felis* containing 1 mg protein, 20 mice were each given orally 0.2 ml of a suspension containing 10 ug of cholera toxin and a sonicate of *H. pylori* (strain 921023) containing 1 mg protein, and 20 mice were not orally vaccinated.

10

One week after the final immunising dose all the mice were euthanased. Samples of antral mucosa were tested for infection using a rapid microtitre urease test as described previously (Lee *et al.*, 1990). This test has been validated as being highly predictive of *H. felis* gastric infection.

15

The results are shown in Table 2.

These results show that oral administration of Helicobacter antigens derived from either *H. felis*, or *H. pylori* along with a mucosal adjuvant, will cure a significant portion of *H. felis* infected mice.

20

TABLE 2

Vaccine	Number of animals infected	Significance
Nil	16/20	
CT alone	15/20	N.S.
<i>H. felis</i> sonicate alone	12/20	N.S.
<i>H. felis</i> sonicate plus CT	8/19	P<0.05*
<i>H. pylori</i> sonicate plus CT	4/20	P<0.001

* Fisher's exact test (two tailed)

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EXAMPLE 3

One hundred female SPF mice from the Animal Breeding Unit of the University of New South Wales, Australia, were infected with 4 oral doses of 10^9 -
5 10^{10} living *Helicobacter felis* (ATCC culture 49179) given 2 days apart. 20 female SPF mice were left uninfected, as negative controls.

Bacteria were grown in plastic Petri dishes on Blood Agar Base No. 2, 3.8% w/v (Oxoid, Basingstoke, UK) with 7% v/v whole horse blood (Oxoid),
10 containing amphotericin B (Fungizone, Squibb, Princeton, N.J., USA) 2.5 mg/l; trimethoprim (Sigma, St.Louis, MO, USA), 10 mg/l. Plates were incubated in a microaerophilic humid atmosphere (Oxoid, BR56) at 37°C for 48 hours.

Sonicates were prepared by growth of the organisms, as described above,
15 followed by harvesting of the organisms in 0.1 molar phosphate buffered saline (PBS). The cells were washed, collected by centrifugation, washed once in PBS, and resuspended in fresh PBS. The cells were then sonicated at the rate of one per minute per ml of cell suspension (50% duty cycle) using a B-30 Branson Cell Disrupter. The sonicate was stored at -20°C.

20 Starting between 6 weeks and 9 weeks after their last infecting dose of *H. felis*, 20 mice were each given orally 0.2 ml of a solution containing 25 µg of *E.coli* heat labile toxin (HLT) (Sigma E 8015), 20 mice were each given orally 0.2 ml of a suspension containing 25 µg of HLT and a sonicate of *H. pylori* containing
25 1 mg protein (BIO-RAD DC protein assay), 20 mice were each given orally 0.2 ml of a suspension containing a sonicate of *H. pylori* containing 1 mg protein, and 40 mice were not orally vaccinated.

Each group received three further doses 15, 17 and 20 days after their
30 initial dose.

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Four weeks after the final immunising dose all the mice were euthanased. Samples of antral mucosa were tested for infection using a rapid microtitre urease test as described previously (Lee *et al.*, 1990). This test has been validated as being highly predictive of *H. felis* gastric infection.

5

The results are shown in Table 3.

They show that oral administration of *Helicobacter* antigens derived from *H. pylori* along with a mucosal adjuvant *E.coli* heat labile toxin, will cure a
10 significant portion of *H. felis* infected mice.

TABLE 3

Treatment Group	Proportion of <i>H. felis</i> infected mice.
Uninfected, unvaccinated	0/20
Infected, unvaccinated	40/40
Infected, Hp antigen alone	20/20
Infected, <i>E.coli</i> HLT alone	20/20
Infected, Hp antigen & HLT	6/19*

* $P < 0.0001$ (Fisher's exact test, two tailed).

REFERENCES:

- Blaser, M.J. (1992). *Helicobacter pylori* : Its role in disease. *Clin. Infect. Dis.* **15**, 386-393.
- Burke, D.S. (1993). Of postulates and peccadilloes : Robert Koch and vaccine (tuberculin) therapy for tuberculosis. *Vaccine* , **11**, 795-804.
- Cox, J. and Coulter, A. (1992). Advances in Adjuvant Technology and Application. *In* Animal Parasite Control Utilising Biotechnology. Edited W.K. Yong, CRC Press.
- Czinn, S.J., Cai, A. and Nedrud, J.G. (1993). Protection of germ-free mice from infection by *Helicobacter felis* after active oral or passive IgA immunization. *Vaccine* , **11**, 637-642.
- Dick-Hegedus, E. and Lee, A. (1991). Use of a mouse model to examine anti-*Helicobacter pylori* agents. *Scand. J. Gastroenterol.* **26**, 909-915.
- Goodwin, C.S. (1993). Overview of *Helicobacter pylori* gastritis, peptic ulcer, and gastric cancer and the possible development of an *H. pylori* vaccine. *In* *Helicobacter pylori* Biology and Clinical Practice. Edited by Goodwin and Worsley. CRC Press.
- Lee, A., Fox, J.G., Otto, G. and Murphy, J. (1990). A small animal model of human *Helicobacter pylori* active chronic gastritis. *Gastroenterology* , **99**, 1316-1323.
- O'Connor, H.J. (1992). Eradication of *Helicobacter pylori* : Therapies and clinical implications. *Postgrad. Med. J.* **68**, 549-557.
- Parsonnet, J., Friedman, G.D., Vandersteen, D.P., Chang, Y., Vogelstein, H.J., Orentreich, N. and Sibley, R.K. (1991). *Helicobacter pylori* infection and the risk of gastric carcinoma. *N.Engl. J. Med.* **325**, 1127-1131.
- Varga, L., Lócsei, Z., Döbrönte, Z., Lakatos, F., Brözik, M. and Meretey, K. (1992). *Helicobacter pylori* allergy. *Orv. Hetil.* **133**, 359-361.

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CLAIMS.

1. A method for the treatment of *Helicobacter* infection in a mammalian host, which comprises the oral administration to said infected host of an immunologically effective amount of one or more *Helicobacter* antigen(s), optionally in association with a mucosal adjuvant.
2. A method according to claim 1, wherein said *Helicobacter* antigen is *H. pylori* antigen.
3. A method according to claim 1, wherein said *Helicobacter* antigen is *H. felis* antigen.
4. A method according to claim 1, wherein said *Helicobacter* antigen(s) comprises a sonicate of *Helicobacter* cells, preferably *H. pylori* cells.
5. A method according to claim 1, wherein said *Helicobacter* antigen is administered in association with a mucosal adjuvant.
6. A method according to claim 5, wherein said mucosal adjuvant is cholera toxin or *E.coli* heat labile toxin.
7. A method according to claim 1, wherein said infected host is an infected human.
8. A vaccine composition for the treatment of *Helicobacter* infection in a mammalian host comprising an immunogenically effective amount of one or more *Helicobacter* antigen(s), optionally in association with a mucosal adjuvant, together with a pharmaceutically acceptable carrier or diluent.
9. A vaccine composition according to claim 8, further comprising an effective amount of a mucosal adjuvant.

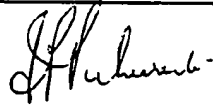
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10. A vaccine composition according to claim 8, wherein said mucosal adjuvant is cholera toxin or *E.coli* heat labile toxin.
11. A vaccine composition according to claim 8, wherein said *Helicobacter* antigen is *H. pylori* antigen.
12. A vaccine composition according to claim 8, wherein said *Helicobacter* antigen is *H. felis* antigen.
13. A vaccine composition according to claim 8, wherein said *Helicobacter* antigen(s) comprises a sonicate of *Helicobacter* cells, preferably *H. pylori* cells.
14. Use of a vaccine composition comprising an immunologically effective amount of one or more *Helicobacter* antigen(s), optionally in association with a mucosal adjuvant, in the treatment of *Helicobacter* infection in a mammalian host.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 94/00416

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁶ A61K 39/02, 39/108 According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int. Cl. ⁶ A61K 39/02 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) CAS DERWENT					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.			
P,X	AU 55619/94 (FONDATION POUR LA RECHERCHE DES MALADIES GASTRO-INTESTINALES : GASTROFONDS MANDATRIA FIDUCIAIRE SA) 11 May 1994 (11.05.94)	1, 7, 10, 14			
P,X	WO 93/20843 (CZINN S.J and NEDRUD J.G.) 28 October 1993 (28.10.93)	1, 7, 8, 14			
P,X	WO 93/16723 (VANDERBILT UNIVERSITY) 2 September 1993 (02.09.93)	8, 11			
X	DE 4139840 (QUIDEL CORP) 11 June 1992 (11.06.92)	8			
<div style="display: flex; justify-content: space-between; align-items: center;"> <div style="display: flex; align-items: center;"> <input type="checkbox"/> Further documents are listed in the continuation of Box C. </div> <div style="display: flex; align-items: center;"> <input checked="" type="checkbox"/> See patent family annex. </div> </div>					
<table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%; vertical-align: top;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 33%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </td> <td style="width: 33%;"></td> </tr> </table>			<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>	
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Date of the actual completion of the international search 5 September 1994 (05.09.94)		Date of mailing of the international search report 20 Sept 1994 (20.09.94)			
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  J.P. PULVIRENTI Telephone No. (06) 2832253			

Information on patent family members

International application No.

PCT/AU 94/00416

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Patent Document Cited in Search Report		Patent Family Member	
AU	55619/94	WO	9409823
WO	9320843	WO	9320843
WO	9316723	AU	37282/93
DE	4139840	FR	2669929
END OF ANNEX			



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(21) International Application Number: PCT/US93/06270 (22) International Filing Date: 1 July 1993 (01.07.93) (30) Priority data: 07/909,382 6 July 1992 (06.07.92) US (71) Applicants: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02139 (US). VIRUS RESEARCH INSTITUTE [US/US]; 61 Moulton Street, Cambridge, MA 02138 (US). (72) Inventors: MEKALANOS, John, J. ; 78 Fresh Pond Lane, Cambridge, MA 02138 (US). BEATTIE, David ; 10 Naponset Court, Boston, MA 02131 (US). KILLEEN, Kevin ; 1112 Brook Road, Milton, MA 02186 (US). LU, Yichen ; 15 South Woodside Avenue, Wellesley, MA 02181 (US).		(74) Agent: FREEMAN, John, W.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US). (81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, LK, MN, NO, NZ, PL, RO, RU, SD, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: DELETION MUTANTS AS VACCINES FOR CHOLERA (57) Abstract The invention features of nontoxicogenic, genetically stable mutant strains of <i>V. cholerae</i> and a method of making which are useful as live, oral vaccines for inducing immunological protection against cholera. The mutant strains are genetically engineered mutants which lack DNA encoding a functional ctxA subunit which is responsible for many of the symptoms of cholera. The strains also lack any functional attRS1 sequences which are required for recombination and amplification of the CTX genetic element. These strains are safe because they can not recombine with the wild type attRS1-containing vehicles which include the ctxA-encoding DNA.		

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DELETION MUTANTS AS VACCINES FOR CHOLERABackground of the Invention

The field of invention is *Vibrio cholerae* vaccines. After more than 100 years of research on cholera, there remains a need for an effective cholera vaccine. There have been six pandemics of this disease caused by strains of *V. cholera* belonging to the "Classical" biotype. The etiological agents of the current (seventh) pandemic belong to the "El Tor" biotype (Finkelstein, Crit. Rev. Microbiol 2:553-623, 1973, Wachsmuth et al., The Lancet 337:1097-1098, 1991). Recently the seventh pandemic has extended to a new locale, that of South America. Beginning in January of 1991, an epidemic of cholera resulted in greater than 250,000 cases and over 2,000 deaths in Peru, Ecuador, Columbia, and Chile. Before this epidemic it was estimated that over 200,000 cases of cholera occurred per year mainly in India, Bangladesh, Africa and Western Asia (Tacket et al., Cholera Vaccines. In Vaccines: New Approaches to Immunological Problems, Ellis, R. W., editor, Butterworth-Heinemann, Boston, 1992).

In November of 1992, an antigenically distinct, non-01 form of *V. cholerae* emerged in India and Bangladesh and within eight months caused an estimated 500,000 cases and 6,000 deaths. The pandemic potential of this new strain, designated serogroup 0139 synonym "Bengal", seems assured and is a new cause of concern throughout the developing world. These recent experiences underline the need for effective cholera vaccines against disease due to both El Tor 01 and Bengal 0139 serotypes of *V. cholerae*.

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Because natural infection by and recovery from cholera induces immunity lasting at least 3 years (Tacket et al., *Supra*; Levine et al., *J. Infect. Dis.* 143:818-820, 1981; Cash et al., *J. Infect. Dis.* 130:325-333, 1974), much effort has been made to produce live, attenuated cholera vaccines that when administered orally would mimic the disease in its immunization properties but would not cause adverse symptoms or reactions in the immunized individual (i. e., display low reactogenicity).

10 Vaccines of this type involve deletion mutations that inactivate the gene encoding the A subunit of cholera toxin, a protein which is responsible for most of the diarrhea seen in this disease (Mekalanos et al., *Proc. Natl. Acad. Sci. USA* 79:151-155, 1982; Mekalanos et al., 15 *Nature* 306:551-557, 1983; Kaper et al., *Nature* 308:655-658, 1984; Kaper et al., *Biotechnology* 2:345, 1984; Pierce et al., *Infect. Immun.* 55:477-481, 1987; Taylor et al., *Vaccine* 6:151-154, 1988; Levine et al., *Infn. Immun.* 56: 161-167, 1988; Herrington et al. *J. Exper.* 20 *Med.* 168:1487-1492, 1988; Levine et al., *Lancet* ii:467-470, 1988; Kaper et al., *Res. Microbiol.* 141:901-906, 1990; Pearson et al., *Res. Microbiol.* 141:893-899, 1990). See also Mekalanos, U.S. Patent Nos. 5,098,998 and 4,882,278, and Kaper et al., U.S. Patent No. 25 4,935,364, hereby incorporated by reference. While both oral, killed whole cell vaccines and several live, attenuated cholera vaccine have been developed, the most promising of these provide little protection against the El Tor biotype of *V. cholerae* and probably no protection 30 against the 0139 serotype. The major issues associated with cholera vaccines are safety, stability and their degree of antigenicity.

With regard to the toxin genes of *V. cholerae*, the genetic diversity among toxigenic and non-toxigenic 35 strains has been examined by Chen et al. (1991,

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Epidemiol. Infect. 107:225). Mekalanos (1983, Cell 35:253) reports on the duplication and amplification of *V. cholerae* toxin genes, and Miller et al. (1984, Proc. Natl. Acad. Sci. USA 81:3471) discusses transcriptional
5 regulation of the toxin genes. Other *V. cholerae* genes whose products may play a role in the pathogenicity of this organism include the toxin-coregulated pilus genes (Shaw et al., 1990, Infect. Immun. 58:3042; Sharma et al., 1989, Vaccine, 7:451; Sun et al., 1990, J. Infect.
10 Dis. 161:1231; Hall et al., 1991, Infect. Immun. 59:2508; Taylor et al., 1987, Proc. Natl. Acad. Sci. USA 84:2833), and the gene encoding the intestinal colonization factor (Taylor et al., 1988, Vaccine 6:151).

Summary of the Invention

15 The invention features a nontoxigenic genetically stable mutant strains of *V. cholerae* which are useful as a live, oral vaccines for inducing immunological protection against cholera. The mutant strains are genetically engineered mutants which lack DNA encoding a
20 functional ctxA subunit and also lack any functional attRS1 sequences. By attRS1 sequences is meant a 17 base pair sequence contained within the CTX genetic element that is required for recombination and amplification of the CTX genetic element, or enough of that sequence to
25 enable such recombination and amplification. Mutants which "lack any functional attRS1 sequences" are those which substantially cannot undergo effective site-specific recombination with attRS1-containing vehicles, because the wild type attRS1 sequences are wholly deleted
30 or are sufficiently deleted or mutated to prevent such recombination. As a result, *V. cholerae* strains according to the invention are safer because they cannot recombine with wild type attRS1-containing vehicles which include the ctxA-encoding DNA.

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The invention also features a method of making the above described *V. cholerae* strains. The method involves introducing a plasmid into a wild type *V. cholerae* which contains a fragment of *V. cholerae* DNA containing a
5 mutation in the *ctxA* and *attRS1* sequences. The *V. cholerae* DNA fragment is capable of recombining with wild type *V. cholerae* DNA inside the organism to generate the mutant strain.

Although any serotype of *V. cholerae* may be used,
10 in preferred embodiments, the mutant strain of *V. cholerae* belongs to the El Tor serotype, and more preferably, the Inaba or Ogawa serotype or the *V. cholerae* non-01 serotype, preferably 0139 "Bengal" serotype. Preferably, the mutants lack all of the CTX
15 core and *attRS1* sequences and more preferably the mutant strain is Peru-2, Bang-2, Bah-2, or an attenuated derivative of the Bengal serotype, such as Bengal-2 ("Beng-2") or Bengal-3 ("Beng-3") as described below.

Mutant strains according to the invention
20 optionally include additional mutations introduced to improve the safety and/or the immunogenicity of the vaccine. Such additional mutations include, but are not limited to, inactivation of one or more genes involved in DNA recombination, for example the *recA* gene encoded by
25 the strain, and the introduction of additional genes which may be introduced into the *V. cholerae* chromosome, preferably into the *V. cholerae lacZ* gene. Preferred additional genes include a gene encoding the B subunit of *V. cholerae* or any heterologous antigen such as the B
30 subunit of Shiga-like toxin, or a gene encoding the *E. coli* CFA antigen, or an antigenic HIV antigen. By heterologous antigen is meant any antigen that is not normally expressed by *V. cholerae*. For example, the heterologous antigen may be *Shigella* lipopolysaccharide
35 (LPS) antigen, Shiga-toxin, various CFA antigens of

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enterotoxigenic *E. coli* strains, anthrax toxin, *Pseudomonas* endotoxin A, antigenic fragments from the HIV capsid, pertussis toxin, tetanus toxin; antigens from Herpes virus, rubella virus, influenza virus, mumps virus, measles virus, poliomyelitis virus; and immunogenic polypeptides from eukaryotic parasites causing malaria, pneumocystis pneumonia, and toxoplasmosis, may be expressed in a *V. cholerae* live vaccine. Preferably, the mutant strain having additional mutations is Peru-14, Peru-3, Peru-4, Peru-5, Bang-3, Bang-5, Bah-3, Bah-4, Bah-5 or an attenuated derivative of Bengal.

By a ctxA subunit is meant the A subunit of the cholera toxin which is responsible, when functional, for many of the symptoms of cholera (e.g., nausea, diarrhea etc.). Most preferably, the strains include deletion of the entire so-called "core genetic element", includes not only the ctxA/B, but also a region known as ICF (Intestinal Colonization Factor, probably equivalent CEP "core encoded pilin") and ZOT, described in greater detail below.

In another aspect, the invention features a nontoxigenic genetically stable mutant strain of *V. cholerae* which is useful as a live, oral vaccine for inducing immunological protection against cholera. The mutant strain is a genetically engineered mutant which lacks DNA encoding a functional ctxA subunit. The strain may also be soft agar penetration-defective. By soft agar penetration-defective is meant lacking the ability to penetrate a media of high viscosity as measured *in vitro* by swarming on and within agar media which is between 0.25 and 0.4% agar. The preferable strain may also be fillamentous, i.e. 25% or more cells greater than 15 nM in length under conditions of logarithmic growth. In preferred embodiments the strain is also ATT-.

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In preferred embodiments, the invention includes a vaccine comprising at least two different strains of *V. cholerae* which are nontoxigenic genetically stable mutants which lack DNA encoding a functional ctxA subunit
5 and are also soft agar penetration-defective. One of the two strains is preferably derived from the Peru strain and the other one is derived from the Bengal strain. The invention also includes a vaccine in which each of the component strains are ctx⁻, att⁻, and recA⁻. Depending
10 upon the relevant local epidemiology, the vaccine strains may be administered together in a single dose, or more preferably, separately 7-28 days apart. Where only one of the serotypes presents a threat of disease, it may be preferable to administer a vaccine regime comprising only
15 one strain.

The invention also features a killed, oral cholera vaccine comprising at least a first and a second *V. cholerae* strain, wherein at least two of the strains are different serotypes and all strains in the mixture lack
20 DNA encoding a functional ctxA subunit. The vaccine also contains cholera toxin B subunit produced by at least one of the serotypes. Preferably, one of the serotypes in the vaccine is an Ogawa serotype and another of the serotypes is an Inaba serotype. Most preferably, the
25 killed oral vaccine comprises Bah-3 and either Peru-3 or Bang-3, or both Peru-3 and Bang-3, as defined below. Any of the oral vaccine combinations may also include cells of the Bengal serotype, as defined below, including Bengal-2 and Bengal-3. The strains may be administered
30 singly, together, or in consecutive doses 7-28 days apart.

The invention also features a method of making a killed *V. cholerae* vaccine. The method involves growing at least a first and a second *V. cholerae* strain, wherein
35 each strain in the mixture lacks DNA encoding a

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functional ctxA subunit. The strains are then collected from the growth medium and the cells are killed. Cholera toxin B subunit, produced by at least one of the strains is obtained from the medium in which the strain was
5 propagated and is added to the killed cells. The mixture of killed bacteria and cholera toxin B subunit is then suspended in a physiologically acceptable carrier.

Mutants such as those described above are useful as cholera vaccines and are improved in their genetic
10 properties compared with previous vaccines.

Other features and advantages of the invention will be apparent from the following description of preferred embodiments thereof, and from the claims.

Detailed Description

15 The drawings will first be briefly described.

The Drawings

Fig. 1. is a schematic diagram of the CTX genetic elements of toxigenic *V. cholerae* strains P27459-Sm, C6709-Sm and E7946-Sm. The filled in boxes represent RS1
20 sequences. Between the RS1 sequences is a region shown as an open box (called the core region) which contains the ctxAB genes and genes encoding zot, the intestinal colonization factor (ICF). At the ends of the RS1 sequences are filled in circles that represent copies of
25 sequences that match 16 out of 17 bases with the 17 base pair sequence attRS1 (CCTAGTGCGCATTATGT) [SEQ.ID.NO:1]. Although the CTX elements of the three strains vary in their structure based on the number of copies of the RS1 and core regions, it should be noted that these elements
30 are inserted into the same chromosomal site in all El Tor strains of *V. cholerae*.

Fig. 2. (A) Restriction map of the chromosome containing the CTX region from strain C6709-Sm with the CTX element schematically shown as in Fig. 1. Not shown

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are the restriction maps of strain P27459-Sm and E7946-Sm which are the same except for the variation observed in sites that map within the CTX element's core or RS1 sequences as designated schematically in Fig. 1. (B)

- 5 Restriction map of corresponding chromosomal region of strain Bang-1, Bah-1, and Peru-1.

Fig. 3. (A) Restriction map of plasmid pGP60 that carries an inserted DNA fragment corresponding to the chromosome containing the CTX region from strain
10 P27459-Sm with the CTX element schematically shown as in Fig. 1. Below this is a two headed arrow which designates the DNA which has been deleted in plasmid pAR62. (B) The restriction map of the CTX region of strain P27459-Sm is shown including restriction sites
15 that map outside the region cloned on plasmid pGP60. (C) A demonstration of the recombinational events (broken lines) between plasmid pAR62 and the chromosome that produced the Type-2 deletion which gave rise in parental strains C6709-Sm, P27459-Sm and E7946-Sm to deletion
20 mutants Peru-2, Bang-2, and Bah-2, respectively. (D) Restriction map of the chromosome of strains Peru-2, Bang-2, and Bah-2.

Fig. 4 is a diagrammatical representation of the construction of plasmid pGP52.

- 25 Fig. 5 is a diagrammatical representation of the generation of pJM84.1 and pJM84.2. A 0.6 kb fragment encoding a promoterless B-subunit was generated by PCR. This DNA was ligated into pCR100 and digested with SpeI/EcoRI. The resulting 0.6 kb restriction fragment
30 was ligated into EcoRI/XbaI digested pVC100 and pRT41 vectors, yielding pJM1001 and pJM411, respectively. Each plasmid was digested with BamHI/EcoRI, treated with Klenow, flanked with XbaI linkers, and digested with XbaI. Purified fragments were ligated to XbaI digested
35 pGP84, yielding pJM84.1 and pJM84.2.

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Fig. 6 is a diagrammatical representation of the insertion of the *ctxB* into the chromosome. Non-replicative pJM84.1 was integrated into Peru-2, Bang-2 or Bah-2 by homologous recombination. Ampicillin resistant
5 recombinant colonies were subsequently plated on medium which contained streptomycin without ampicillin, thus reducing the selective pressure for ampicillin resistance. The resulting ampicillin sensitive colonies were isolated and had selected for excision of DNA
10 flanked by homologous *recA* DNA sequences.

The invention features attenuated strains of *V. cholerae* that can be used either as live or killed oral vaccines to protect individuals against cholera and potentially other diseases.

15 Construction of Vaccines

Attenuated derivatives of a *V. cholerae* strain C6709-Sm isolated from a cholera patient in Peru in 1991 have been constructed that can be used as live, oral cholera vaccines. The derivatives Peru-1 and Peru-2,
20 carry small Type-1 (core) and large Type-2 deletions, respectively, which remove the DNA encoding the cholera toxin in addition to DNA encoding *zot*, an intestinal colonization factor (ICF) that is unrelated to cholera toxin. Because excessive intestinal colonization may be
25 responsible for adverse side effects seen in humans administered earlier prototype live cholera vaccines, the deletion of genes encoding both cholera toxin and ICF in Peru-1 and Peru-2 will render these strains less reactogenic in vaccinees while they retain their
30 immunogenic and therefore protective properties.

The larger Type-2 deletion present in Peru-2 also removes an insertion-like sequence called RS1 which is present in two or more copies as part of a larger DNA

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segment called the CTX genetic element. The RS1 sequence encodes a site-specific recombination system that can duplicate at a high frequency and cause insertion of the CTX element into the *V. cholerae* chromosome at a 17 base pair target site called *attRS1*. Sequences nearly identical to *attRS1* (and apparently just as recombinationally active) exist at the ends of the RS1 sequences. These sequences are as follows:

attRS1 and flanking chromosomal sequences:

10 5'-TAAACCTAGAGACAAAATGTTCCTAGTGCGCATTATGTATGTTATGTTAAAT-3'
[SEQ.ID.NO:2]

Left side of RS1 and chromosomal junction:

5'-TAAACCTAGAGACAAAATGTTCCTAGTGCGCATTATGTGGCGCGGCAT...RS1...-3'
[SEQ.ID.NO:3]

15 Right side of RS1 and chromosomal junction:

5'-AAACCTAGATTCCGCGCCTTAGTGCGCATTATGTATGTTATGTTAAAT-3'
[SEQ.ID.NO:4]

The *attRS1* and a similar sequence present at the ends of RS1 are underlined. Note that the chromosomal sequence that flanks *attRS1* is present on the left and the right side of RS1 with the only overlap being a 17 base pair sequence that is identical to *attRS1* on the left end of RS1 and an 18 base pair sequence that matches 17/18 base pairs with *attRS1*.

25 Genetically engineered live attenuated cholera vaccines are theoretically safe only if they cannot revert or otherwise regain the capacity to produce cholera toxin. Strains which carry a single copy of the *attRS1* sequence can efficiently acquire a new copy of the CTX element through DNA transfer by either P factor conjugation or bacteriophage transduction. Thus, deletions which render *V. cholerae* devoid of RS1 and *attRS1* sequences can prevent a vaccine strain from reacquiring the CTX genetic element in nature through its own site specific recombination system. Such a deletion is present in strain Peru-2 and its derivatives.

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Six mutant strains of *V. cholerae* with similar but not identical properties have been constructed. Four strains that carry the same two types of deletions (Type-1 and Type-2) as strains Peru-1 and Peru-2 were
5 constructed in *V. cholerae* strains isolated from patients in Bangladesh (P27459-Sm) and Bahrain (E7946-Sm). These four derivatives, Bang-1, Bang-2, Bah-1 and Bah-2 are also the subject of the invention because they vary in colonization and/or other properties (e.g., serotype) and
10 they are therefore potentially more suitable than the corresponding Peru strains for use as vaccines in other areas of the world.

Although the smaller Type-1 deletion present in the three strains Peru-1, Bang-1 and Bah-1 does not
15 remove all copies of RS1, this particular deletion affects the intestinal colonization properties of some of these strains more severely than the larger deletion present in Peru-2, Bang-2 and Bah-2.

Construction of Type-2 Deletion Mutations

20 A Type-2 deletion removes all sequences corresponding to the CTX genetic element including RS1 sequences and all copies of the attRS1 sequence (Fig.1). The Type-2 deletion was constructed by recombination between the chromosome of *V. cholerae* and the plasmid
25 sequences cloned on plasmid pAR62 as shown in Fig. 3. Plasmid pAR62 is a derivative of plasmid pGP60 and carries a Type-2 deletion wherein the HindIII fragment shown in Fig. 3 was deleted. Plasmid pGP60 was constructed by first generating a genomic library of
30 strain P27459 by inserting 20-30 kb Sau3A partially digested fragments into the BamHI site of plasmid pLAFR2 (Friedman et al., 1982, Gene 18:289). Colonies were screened by hybridization using probes derived from the ctx region (Mekalanos, 1983, Cell 35:253). A positive

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colony was picked and the plasmid which was isolated therefrom was named pGP60. Restriction enzyme analysis of this plasmid confirmed that it contained all of the CTX element sequences and additional flanking DNA.

5 Plasmid pAR62 encodes resistance to tetracycline. This plasmid was introduced into a strain of *V.cholerae* by conjugation or electroporation followed by selection on media containing 3 μ g/ml of tetracycline. Such a plasmid carrying strain was then screened by colony hybridization
10 with radioactive L-3 probe prepared as described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986). Colonies carrying the Type-2 deletion inserted into the chromosome did not hybridize to the L-3 probe and surprisingly, occurred at a high frequency (i.e.,
15 about 1% of the colonies screened). Southern blot analysis was used to confirm the presence of the expected deletions in these strains.

Construction of Core (Type-1) Deletions

A "core deletion" removes only sequences
20 corresponding to the core of the CTX element but leaves behind a copy of the RS1 element on the chromosome (Goldberg et al., J. Bacteriol. 165:723-731, 1986) (Fig. 2.). These deletions occur spontaneously through homologous recombination between RS1 sequences located on
25 the right side and left side of the core region as shown in Fig. 2. Colonies of *V. Cholerae* that contain core deletions can be identified in two ways. First, if the strain carries a selectable marker such as a gene encoding kanamycin resistance inserted in the core
30 region, then the core deletion renders such a strain sensitive to kanamycin (Goldberg et al., J. Bacteriol. 165:723-731, 1986). Second, colonies that contain the core deletion can also be identified by colony hybridization using radioactive CT-1 probe which does not
35 hybridize to strains carrying this deletion (Goldberg et

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al., J. Bacteriol. 165:723-731, 1986). By either method, colonies that carry these deletions occurred at a frequency of about 1 per 1000 colonies screened.

Analysis by Southern blot hybridization was then used to
5 confirm the expected deletions in these strains.

An Assay for Functional attRS1 Sequences Based Upon
Integration of Plasmid pGP52

The plasmid pGP52 is a suicide plasmid which is only capable of replicating in strains of *E. coli* such as
10 SM10 λ pir (Pearson et al., 1990, Res. Microbiol. 141:893). Plasmid pGP52 was constructed by first digesting the plasmid pGP7 (Mekalanos, 1983, Cell 35:253) with ClaI and SphI. This plasmid contains two RS1 sequences (termed RS1 and RS2) derived from the *V. cholerae* strain E7946-
15 Sm. A fragment of DNA which contained the RS1 sequences was cloned into pBR322 and the resulting plasmid was named pGP20. This plasmid was then digested with EcoRV (which cuts within the RS1 sequences). When this plasmid was religated a new plasmid termed pGP20R was generated
20 containing a hybrid version of RS2 called RS2*, wherein the hybrid RS2 sequences were flanked by core sequences. An SspI-SphI fragment of RS2 was then subcloned into the suicide plasmid pJM703.1 which had been digested with NruI and SphI. The plasmid pJM703.1 is described in
25 Miller et al. (Proc. Natl. Acad. Sci. USA 81:3471). The resulting plasmid was called pGP52. A diagram depicting the construction of pGP52 is shown in Fig. 4.

When pGP52 is transferred by conjugation into *V. cholerae* strains which contain attRS1 sequences, it
30 integrates into the *V. cholerae* chromosome by means of a site-specific recombination event between the attRS1 sequence on the chromosome and the attRS1 sequence present on the plasmid. Integration events such as these can be quantitated by determining the number of colonies
35 that stably maintain (i.e., are non-selected) ampicillin

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resistance because resistance to ampicillin is encoded by pGP52. Confirmation of integration can be obtained in Southern blot hybridization experiments. If the *V. cholerae* strain to be tested has functional attRS1 sequences then integration will be observed in the test. If the strain does not contain functional attRS1 sequences, integration will not occur.

In order to assess the ability of the various vaccine candidates to serve as recipients for pGP52, the following experiments were performed. Donor *E. coli* strain SM10 λ pir pGP52 was mixed with the recipient *V. cholerae* test vaccine strain in 5 ml of Luria broth at concentration of 10^7 cells from each strain per culture. The mixture was incubated at 37°C for 5 hours at which time it was diluted 1:100 into fresh Luria broth containing 100 μ g/ml of streptomycin. The purpose of the streptomycin is to select against the *E. coli* donor strain by killing it. Thus, only the streptomycin resistant *V. cholerae* recipient strains are capable of growth. This culture was incubated until the growth rate of the cells reached saturation. The cultures were diluted again and further incubated until each cell had replicated a total of 20 times in the absence of any positive selection for pGP52. This culture was then diluted and plated on two separate media compositions in order to quantitate the number of viable colonies. One of these media is Luria broth which does not contain any antibiotics. The number of colonies appearing on these plates represents the total number of cells in the culture. The other medium is Luria broth which contains ampicillin. The number of colonies appearing on these plates represents the number of integration events that occurred following conjugation. The results are expressed as a ratio of stable integration events/total

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number of viable cells and are presented in Table 1 below.

Table 1. Representative Integration Data on Peru Vaccine Strains

<u>Strain</u>	<u>Stable Integration events/total # viable cells</u>
5 Peru-1	5.2×10^{-5}
Peru-2	Not detectable ($< 5 \times 10^{-8}$)
Peru-3	Not detectable ($< 5 \times 10^{-8}$)
Peru-4	Not detectable ($< 5 \times 10^{-8}$)
Peru-5	Not detectable ($< 5 \times 10^{-8}$)

10 Based on these data it is evident that strain Peru-1, which contains two copies of the attRS1 sequences is capable of integrating the plasmid pGP52 into its chromosome at a frequency that is at least 1000-fold higher than any of the other strains tested, all of which
15 lack any attRS1 sequences.

Serological Characterization of Vaccine Strains

The vaccine strains Peru-2, Bang-2, and Bah-2 were characterized further in terms of their serological and colonization properties. The data presented in Table 2
20 demonstrate that each derivative retained its expected serotype (i.e., the serotype of each of the mutants respective parental strain) when freshly harvested bacterial cells were tested by slide agglutination using Difco *V. cholerae* 01 Inaba or Ogawa typing serum. This
25 result indicates that these strains still express LPS antigens. Other tests demonstrate that these mutant strains are motile, prototrophic, and still express Tcp pili. Thus, the mutants express a number of properties that are important for their ability to be useful as live
30 vaccine strains.

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Colonization Properties of the Vaccine Strains and Core Deletion Mutants

To test the colonization properties of these vaccine strains, a mouse intestinal competition assay was used as described in Taylor et al. (Proc. Natl. Acad. Sci. USA. 84:2833-2837, 1987) which has been shown to correlate accurately with the colonization properties of mutant strains when they are subsequently tested in human volunteers (Herrington et al., J. Exper. Med. 168:1487-1492, 1988). The assay measures differences in colonization of a mutant strain by comparing its ability to compete for growth and survival with another closely related or isogenic strain. In this assay, the mutant and competing strains were mixed in a ratio of approximately 1:1 and then approximately one million cells of this mixture were introduced to the stomach of 3-5 day old suckling CD-1 mice. After 24 hours, the mice were sacrificed, the intestine was dissected, homogenized, and plated on bacteriological media containing streptomycin which selects for both strains. Colonies that grew after overnight incubation are then tested for additional markers which differentiate the mutant strain from the competing strain (i.e., resistance to kanamycin or hybridization with appropriate radioactive DNA probes; see legend of Table 3).

As shown in Table 3, Bang-2, and Bah-2 both exhibited a mild intestinal colonization defect that resulted in approximately 4-13 fold greater recovery of the isogenic competing strains than the mutant strains after 24 hours of growth in the mouse intestine. Also shown in Table 3, are results from competition assays involving core deletion mutant strains Peru-1, Bang-1 and Bah-1. Like the Type-2 deletion strains Bang-2 and Bah-2, these core deletion mutants were defective in colonization relative to their isogenic competing

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strains. Because core deletions remove sequences corresponding to the core of the CTX element (Fig. 1 and 3), these data suggest that the core of CTX element encodes an "intestinal colonization factor, or ICF".

- 5 Cholera toxin by itself is not an ICF. Strains SM44 and SM115 which are defective in cholera toxin production due to a deletion in the *ctx* genes and insertion of a gene encoding kanamycin resistance as described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986)
- 10 outcompete their respective mutant strains (Bang-1, Bang-2 and Bah-1, Bah-2) in the intestinal competition assay. Thus, it is apparent that SM44 and SM115 make ICF even though they do not produce cholera toxin, while the mutants do not. Furthermore, because the CTX core region
- 15 was the only DNA that is deleted in both core as well as Type-2 deletions and mutants carrying both types of deletions were similarly defective in colonization, it can also be concluded that ICF is encoded by the core region of the CTX element as shown in Fig. 1.
- 20 Recently, a new toxin called ZOT has been found to be encoded by the core region (Baudry et al., 1992, Infect. Immun. 60:428-434). We have evidence that mutations in the *ZOT* gene do not produce the colonization defect observed in Type-1 or Type-2 deletion mutants.
- 25 Accordingly, ICF is designated as a separate and distinct property from ZOT. The vaccine strains described herein carrying Type-1 or Type-2 deletions are defective in ICF.

In contrast, strain Peru-2 exhibited no significant defect in intestinal colonization relative to

30 its competing strain C6709-Sm (Table 2). However, the total cell yield of either strain C6709-Sm or Peru-2 in the mice was typically 10-100 fold less than strains SM44 or SM115, suggesting that the Peru strain C6709-Sm and its derivative Peru-2 may already carry an undefined

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colonization defect. Since deletion of the core of all or part of the CTX element did not cause a further defect in the colonization of either strain Peru-1 or Peru-2, it can be concluded that strain C6709-Sm is partially defective in ICF already even though it carries DNA sequences that correspond to the CTX core region. Deletion of the entire CTX region as defined by the Type-2 mutations present in strains Peru-2, Bang-2 and Bah-2 assures that the genes for ICF cannot reactivate and become functional in the vaccine derivatives. The Type-2 deletion of ICF genes apparently causes a mild colonization defect. Such may be useful as an attenuating mutation in cholera vaccine development, because wild type ICF may be responsible for undesirable levels of toxicity.

Table 2. Properties of Mutant Strains

<u>Mutant Strains</u>	<u>Parental Strain</u> *	<u>Serotype</u>	<u>Type of Deletion</u>
Peru-2	C6709-Sm	Inaba	Type-2
Bang-2	P27459-Sm	Ogawa	Type-2
20 Bah-2	E7946-Sm	Inaba	Type-2

* Note that the designation "Sm" behind the strain name refers to streptomycin resistance. This is a spontaneously selected strain which is resistant to 100 μ g/ml of streptomycin and was the result of a spontaneous point mutation in the gene for a ribosomal protein. This resistance marker is not associated with a plasmid or transposon and is therefore not transmissible to enteric flora. Because all mutant strains are derived from the indicated parental strains, all mutant strains are also resistant to streptomycin.

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Table 3. Infant Mouse Colonization Competition Assays^a

	<u>Mutant Strain</u>	<u>Competing Strain</u>	<u>Input Ratio</u>	<u>Output Ratio</u>
			<u>Mutant/Competing Strain</u>	<u>Mutant/Competing Strain</u>
5	Bang-2	SM44 ^b	0.61	0.16
	Bah-2	SM115 ^c	0.92	0.07
	Peru-2	C6709-Sm ^d	0.74	0.65
	Bang-1	SM44 ^b	0.85	0.05
	Bah-1	SM115 ^c	0.61	0.04
	Peru-1	C6709-Sm ^d	0.89	0.94

- 10 ^a Infant mouse colonization assays were performed according to the method described in Taylor et al. (Proc. Natl. Acad. Sci. USA. 84:2833-2837, 1987). The ratio of strains was determined by either differential sensitivity to antibiotics or by colony hybridization with
- 15 appropriate probes as described in the additional footnotes below.

^bStrain SM44 has been described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986) and is a kanamycin resistant derivative of the parental strain P27459-Sm.

- 20 The gene encoding kanamycin resistance in SM44 was inserted in the *ctx* locus. Because Bang-1 and Bang-2 were derivatives of P27459-Sm competition with SM44 measures colonization differences that can be attributed to the effect of the Type 2 rather loss of *ctx*. Strains
- 25 Bang-1 and Bang-2 were sensitive to kanamycin and were differentiated from SM44 in these competitions assays by scoring colonies for resistance to 30 µg/ml kanamycin.

- ^cStrain SM115 has been described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986) and is the
- 30 kanamycin resistant derivative of the parental strain E7946-Sm. The gene encoding kanamycin resistance in SM115 was inserted in the *ctx* locus. Because Bah-1 and Bah-2 are derivatives of P27459-Sm competition with SM115 measures colonization differences that can be attributed

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to the effect of the Type 2 deletion rather than loss of
ctx. Strains Bah-1 and Bah-2 were sensitive to
kanamycin and were differentiated from SM115 in these
competitions assays by scoring colonies for resistance to
5 30 μ g/ml kanamycin.

^dStrain C6709-Sm is the parental strain of Peru-1 and
Peru-2. Peru-2 carries a Type -2 deletion while Peru-1
carries a core deletion. Both these deletions remove the
ctx genes and thus both Peru-1 and Peru-2 were negative
10 in colony hybridization blots when probed with the CT-1
probe described in Goldberg and Mekalanos (J. Bacteriol.
165:723-731, 1986) while strain C6709-Sm was positive
using the same probe. Thus, both Peru-1 and Peru-2 were
differentiated from C6709-Sm in these competitions assays
15 by scoring colonies for hybridization with the CT-1
probe.

The mutant strains described can be further
improved as vaccine candidates by creating additional
mutations within each strain that will serve to enhance
20 the safety and immunogenicity of the vaccine.

With regard to safety, a second mutation can be
introduced into the *recA* gene of any of the strains
described above, which mutation is designed to inactivate
that *recA* gene. Such double mutant strains will
25 therefore be defective in recombination and will be
unable to recombine with wild type strains of *V. cholerae*
in the environment. Thus, they will be incapable of
acquiring wild type toxin genes and expressing the CTX
element. Immunogenicity can also be improved by
30 introducing additional mutations into each strain which
will allow that strain to express cholera toxin related
antigens (e.g., the B subunit of cholera toxin) or other
heterologous antigens, e.g., the nontoxic B subunit of
Shiga-like toxin or various CFA antigens of

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enterotoxigenic *E. coli* strains, Shiga-toxin, anthrax toxin, *Pseudomonas* endotoxin A, pertussis toxin, tetanus toxin; antigens from Herpes virus, rubella virus, influenza virus, mumps virus, measles virus, poliomylitis virus, antigenic fragments from the HIV capsid; and immunogenic polypeptides from eukaryotic parasites causing malaria, pneumocystis pneumonia, and toxoplasmosis (Karjalainen et al., 1989, Infect. Immun. 57:1126; Perez-Casal et al., 1990, Infect. Immun. 58:3594). Thus, a series of mutated derivatives can also be useful in the invention, each incorporating additional properties that render the strains safer, genetically more stable and more broadly immunogenic. The construction of such derivatives is described below.

15 Construction of recA/ctxB Alleles

Cholera toxin B subunit is known to be a nontoxic, highly immunogenic molecule that is capable of inducing cholera toxin neutralizing antibodies. In order to generate more immunogenic vaccine strains, a new copy of the ctxB gene was introduced into the vaccine strains containing the Type-2 deletions described above (because Type-2 deletions remove all of the coding sequence for the cholera toxin B subunit). This was accomplished in a series of steps that are described below.

25 First, a promoterless copy of the ctxB gene was constructed using the polymerase chain reaction (PCR). For PCR, the downstream primer was designed so that the ctxB coding sequence could be synthesized in such a way as to eliminate the attRS1 site that lies just downstream from the stop codon in the ctxB gene. This primer had the following sequence: 5'-

GGGCTAAAGTTAAAAGACAAATATTTTCAGGC-3' [SEQ.ID.NO:5]. The upstream primer was designed so that only the last 24 carboxyterminal amino acid residues of the A2 subunit

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could be encoded by the product of the reaction. This primer had the following sequence:

5'-GGGTAGAAGTGAAACGGGGTTTACCG-3' [SEQ.ID.NO:6].

All other nucleotides in the DNA encoding the A subunit were excluded from the reaction. The DNA encoding the carboxyterminal amino acids of CtxA2 were retained in the final product to allow for translational coupling of *ctxB* gene expression. Since the toxic activity associated with cholera toxin is derived from the CtxA1 polypeptide, all sequences encoding the A1 polypeptide were excluded from the PCR reaction.

PCR was performed using the *ctxB* primers as described above using *V. cholerae* DNA from the Peruvian strain, C6709-Sm (Fig. 5). The product of the reaction, a 0.6 kilobase pair fragment, was cloned into plasmid pCR100. This fragment was then cut out of the plasmid as a 0.6 kilobase pair *SpeI*-*EcoRI* fragment and was cloned into two individual acceptor plasmids, *XbaI*-*EcoRI* digested pRT41 and *XbaI*-*EcoRI* digested pVC100. The resulting plasmids, pJM411 and pJM1001, then each encode a copy of the *ctxB* gene under the control of either the *ctx* promoter (*ctxP*) or the *htpG* promoter (*hptP*) of *V. cholerae*, respectively. These plasmids were then transferred to the nontoxigenic strain *V. cholerae* 0395-NT (Mekalanos et al., 1983, Nature 306:551 and U.S. Patent No. 4,935,364), generating two new strains termed 0395-NT pJM411 and 0395-NT pJM1001. The amount of cholera B subunit produced by each strain was measured by GMI ELISA. Strain 0395-NT pJM411 produced 30 $\mu\text{g/ml}$, while strain 0395-NT pJM1001 produced 100 $\mu\text{g/ml}$ in LB culture supernatant fluids. These results demonstrate that the PCR product was a functional *ctxB* gene encoding an antigenic cholera B subunit capable of binding to ganglioside GMI and was therefore similar to that secreted by normal wild type *V. cholerae*.

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In the next step, EcoRI-BamHI fragments of DNA specifying the promoter-ctxB constructs were subcloned into the suicide *recA* plasmid pGP84. This plasmid contains a *V. cholerae* chromosomal DNA insert that

5 corresponds to the DNA which flanks the *recA* gene of *V. cholerae* (i.e., an internal deletion of *recA*). Plasmid pGP84 is a derivative of suicide plasmid pJM703.1 (Miller et al., 1988, J. Bacteriol. 170:2575) and encodes sequences corresponding to the flanking regions of the

10 *recA* gene of *V. cholerae* (Goldberg et al., 1986, J. Bacteriol. 165:715) including a BglII-PvuII fragment on the left side and an XbaI-EcoRI fragment on the right side. A 1.3 kb fragment encoding kanamycin resistance is positioned between these two fragments. Plasmid pGP84

15 also contains a NruI-BamHI fragment encoding sensitivity to streptomycin. This latter fragment is derived from plasmid pNO1523 (Dean, 1981, Gene 15:99). When pGP84 is digested with XbaI, the 1.3 kb fragment is removed and other XbaI fragments can be inserted into this deleted

20 *recA* region.

The subcloning was accomplished as follows: Each of the two EcoRI-BamHI fragments specifying the promoter-ctxB constructs were modified by the addition of XbaI linkers. They were individually ligated to XbaI digested pGP84 to

25 generate two new plasmids pJM84.1 and pJM84.2, each of which contains DNA specifying the *htpP-ctxB* and the *ctxP-ctxB* constructs respectively (Fig. 6).

Next, plasmids pJM84.1 and pJM84.2 were transferred into *V. cholerae* strains Peru-2, Bang-2 and

30 Bah-2 and ampicillin resistant colonies were selected. Because these plasmids are incapable of replication in *V. cholerae*, they integrate into the host cell chromosome by homologous recombination generating the structure shown in Fig. 6. Both plasmids also encode a gene for

35 streptomycin sensitivity which allows for positive

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selection against a plasmid integration event in strains that are streptomycin resistant (i.e., strains Peru-2, Bang-2 and Bah-2). Thus, when strains that have a plasmid integrated into the chromosomal DNA are grown on medium containing 2 mg/ml streptomycin, colonies that have reverted to ampicillin sensitivity can be isolated. Strains that had now crossed out the integrated plasmid in such a way as to leave behind the *recA* deletion mutation together with the *ctxB* construct were then selected from among these latter strains. These strains were easily identified as having the following properties:

1. They were ampicillin sensitive.
2. They were killed in the presence of 0.1 ml methyl methane sulfonate per ml of LB, a characteristic phenotype of *recA*⁻ cells.
3. They produced the cholera B subunit as measured by GMI-ELISA.
4. Southern blot analysis using *recA* and *ctxB* probes confirmed that they contained DNA fragments consistent with the presence of the *ctxB* construct and deletion of the appropriate *recA* sequences.

Bacterial strains that were isolated following the procedure described above are as follows:

25	STRAIN	GENOTYPE
	Peru-3	<i>attRS1</i> deletion, <i>recA</i> :: <i>htpP-ctxB</i> , <i>str</i>
	Peru-4	<i>attRS1</i> deletion, <i>recA</i> :: <i>ctxP-ctxB</i> , <i>str</i>
	Bang-3	<i>attRS1</i> deletion, <i>recA</i> :: <i>htpP-ctxB</i> , <i>str</i>
	Bah-3	<i>attRS1</i> deletion, <i>recA</i> :: <i>htpP-ctxB</i> , <i>str</i>
30	Bah-4	<i>attRS1</i> deletion, <i>recA</i> :: <i>ctxP-ctxB</i> , <i>str</i>

Construction of *lacZ-ctxB* Alleles

The *recA* mutation contained within the vaccine strains described above renders the strains deficient in homologous recombination. In order to produce candidate vaccines that were still capable of homologous recombination, the *ctxB* gene was inserted into the *lacZ* gene of *V. cholerae* as described below.

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The plasmid pCG698 which encodes the *lacZ* gene of *V. cholerae*, contains a unique *HpaI* site in the middle of the *lacZ* coding sequence. The plasmid pCG698 was constructed as follows: The β -galactosidase gene of *V. cholerae* was cloned from a library of chromosomal DNA fragments from strain E7946 as described (Mekalanos, 1983, Cell 35:253). It was found to express β -galactosidase and following restriction enzyme mapping, was found to contain a 6 kb insert containing 2 *HpaI* sites in the *lacZ* gene each of which was separated by 2.1 kb of DNA. This plasmid was linearized with *HpaI* and *XbaI* linkers were ligated to the ends. An *EcoRI*-*BamHI* fragment containing the *ctxP-ctxB* construct was removed from pJM411 as described above, the ends were modified by the addition of *XbaI* linkers and the fragment was ligated into the similarly modified pCG698. The resulting plasmid pJM6891, now contained the *ctxP-ctxB* construct inserted into the middle of the *lacZ* gene. This plasmid was transferred into *V. cholerae* strains Peru-2, Bang-2 and Bah-2 and each resulting strain was screened for growth in the presence of X-gal. White colonies containing an inactivated *lacZ* gene were picked and purified. Strains that contained an integrated copy of the *lacZ::ctxP-ctxB* sequences into the host cell chromosome were obtained by curing the bacteria of pJM6891 by growth in the absence of ampicillin. The presence of the appropriate sequences was confirmed by Southern blot analysis and the ability of these bacteria to produce cholera toxin B subunit was confirmed by GMI-ELISA. Bacterial strains isolated following this procedure are as follows:

STRAIN	GENOTYPE
Peru-5	<i>attRS1</i> deletion, <i>lacZ::ctxP-ctxB</i> , str
Bang-5	<i>attRS1</i> deletion, <i>lacZ::ctxP-ctxB</i> , str
Bah-5	<i>attRS1</i> deletion, <i>lacZ::ctxP-ctxB</i> , str

In order to characterize some of these carrier cholera vaccine candidates with regard to mouse

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colonization, mice were infected with the strains listed below. The strain TCP2, a derivative of 0395-N1 which contains a TcpA deletion and does not colonize the intestine of human volunteers, served as a control. Five mice were used for each strain. At 24 hours post-infection, the upper intestine was removed from each mouse, homogenized and assayed for the number of *V. cholerae* present using a simple plating assay. The results are presented in the table below. Essentially, no TCP2 bacteria were detected in the intestines of mice infected with TCP2 and thus the values given below represent the number of bacteria of each strain that colonized the mouse intestine above a background level of zero.

15	Strain	CFU per mouse ^a	Genotype/Construct ^b	
	Peru-3	9.4×10^5	<u>attRS1</u> deletion	#2, <u>recA::htpG-ctxB</u>
	Peru-2	2.5×10^6	<u>attRS1</u> deletion	#2, <u>recA::ctx-ctxB</u>
	Peru-4	6.0×10^6	<u>attRS1</u> deletion	#2, <u>lacZ::ctx-ctxB</u>
	Peru-5	6.6×10^6	<u>attRS1</u> deletion	#2, <u>recA::htpG-ctxB</u>
20	Bang-2	9.9×10^6	<u>attRS1</u> deletion	#2, <u>recA::htpG-ctxB</u>
	Bang-3	2.7×10^7	<u>attRS1</u> deletion	#2, <u>recA::htpG-ctxB</u>

a Colony forming units recovered per mouse (average of five mice).

b The construct attRS1 deletion #2 is a Type 2 deletion constructed with plasmid pAR62, described in Figure 3.

25 The construct recA::htpG-ctxB is a deletion of the recA gene and insertion of the cholera toxin B subunit gene under control of the heat shock promoter derived from the htpG of V. cholerae.

30 The construct recA::htpG-ctxB is a deletion of the recA gene and insertion of the cholera toxin B subunit gene under control of the cholera toxin promoter derived from the ctx gene of a hypervirulent strain 5698 of V. cholerae.

The construct lacZ::ctx-ctxB is an insertion in the lacZ gene of V. cholerae that is composed of the cholera toxin B subunit gene under control of the cholera toxin promoter derived from the ctx gene of a hypervirulent strain 5698 of V. cholerae.

The results suggest that the presence of the recA::htpG-ctxB allele serves to reduce the ability of the Peru-derived strains to colonize the intestine (compare, for example, Peru-3 with Peru-2). However, the effect of this construct on colonization of the Bang-derived strain was less marked (compare Bang-3 with Bang-

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2). In general, introduction of the constructs wherein *ctxB* is under the control of its own promoter had less effect on colonalization that the constructs wherein it was placed under the control of the heat shock promoter.

5 It should be noted that strains Peru-2, Peru-3 and Bang-3 vary in their colonalization properties over a 28-fold range. It is well within the art following the protocols described above, to isolate additional vaccine candidates that vary even more widely in their colonalization

10 properties.

In summary, the data demonstrate the feasibility of using genetic engineering techniques to generate novel *ctxB*-containing *V. cholerae* strains wherein the expression of the *ctxB* gene is placed under the control

15 of either of two *V. cholerae* promoters (*ctxP* and *htpP*). The engineered genes can be recombined into the *V. cholerae* chromosome into target genes such as *recA* or *lacZ* to generate strains which stably express large amounts of cholera toxin B subunit (for example, strains

20 Peru-3, Peru-4 and Peru-5).

Isolation of spontaneous soft agar penetration-defective strains of *V. cholerae*

Mutants of *V. cholerae* which are defective in soft agar penetration can be useful in the production of

25 vaccines. The rationale for utilizing these mutants is as follows. The mucous layer of the intestine is thought to be viscous and mutants defective in penetration of soft agar might be deficient in penetration of this mucous. Although defective in penetration through mucous, these

30 mutants may still present antigen to the Peyer patches which are not covered by a thick mucous gel and which include antigen-sampling cells specific for IgA antibody production. As a result, penetration defective mutants are predicted to have low reactogenecity, yet be highly

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antigenic, and these characteristics are desirable for a live vaccine. Although non-motile mutants are one class of mutants defective in penetration of soft agar, other types of mutations may also result in a soft agar

5 penetration-defective phenotype (i.e., a swarming phenotype) and may be useful for vaccines. In keeping with this line of reasoning, completely non-motile mutants, i.e., mutants unable to swarm in agar-free media, may be useful candidate vaccines.

10 To obtain such mutants, soft agar can be used to assess the ability of bacteria to penetrate a media of high viscosity (soft agar media which is 0.25 - 0.4% agar), as described below. One such soft agar penetration-defective vaccine with a high therapeutic
15 value is Peru-14.

Peru-14 is soft agar penetration-defective, and, in addition, over 50% of Peru-14 cells are fillamentous, with a spiral-like appearance and having a cell length of greater than 5 normal cell lengths (25nM, as opposed to
20 the wild-type cells length of 5nM).

Peru-14 was isolated as a soft agar penetration-defective derivative of the triply-deleted Peru strain (Peru-3) (ctxA⁻, att⁻, and recA⁻) that was free from side effects but still retained the ability to colonize
25 vaccinees as shown in below (Table 7).

Although Peru-14 was isolated based upon the theory stated above, this theory of function may or may not accurately and completely explain the effectiveness of Peru-14 as a vaccine. The usefulness of Peru-14 as an
30 effective vaccine does not depend on the correctness of this theory.

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Table 7**Outcome of Immunization with Freshly Harvested Peru-14
Cholera Vaccine**

of Dose (cfu) 10 ⁶ ay	Volunteer #	Symptoms	Stool	Duration	
				Excretion (days)/Peak	
2x10 ⁶ 3/3	28	Gas	Formed		
15/2	29	Cramps	Formed		
-	30	None	Formed		--
4/4	33	None	Formed		
20 4/1	34	None	336g*		
3/3	35	None	Formed		
9x10 ⁸ 25/1	25	None	Formed		
3/1	26	Gas	Formed		
2/2	27	Headache	Formed		
30 7/4	31	Nausea, Loss of Appetite	Formed		
5/3	32	None	Formed		
35/1	36	Cramps	63g+		

- * Volunteer had painless semi-solid stool at 72 hours post-immunization. Stool was culture-negative for Peru-14.
- 40 + Volunteer had two small liquid stools at 48 hours post-immunization. Stools were culture-positive for Peru-14.

Specifically, the Peru-14 soft agar penetration-defective strain was produced as follows. Peru-3 was grown overnight in LB broth containing 100 µg streptomycin sulfate at 30°C. The culture was diluted to approximately 2000 cfu/ml and 0.1 ml was plated onto LB plates containing 100 µg streptomycin. After incubating the plates overnight at 30°C, approximately 1000 colonies

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were toothpicked into soft agar plates (LB broth + 0.45% Bacto-agar) and incubated overnight at 30°C. The inoculating toothpick is inserted only 1-2 mm into the surface of the soft agar plate. Of the 1000 colonies
5 picked, 25 appeared to be non-penetrating. Non-penetrating isolates appear as colonies of approximately 2 mm in diameter, whereas penetrating isolates swarm on and within agar the agar to a diameter greater than 5 mm. These colonies were repicked into soft agar once again,
10 along with a known non-penetrating, non-motile cholera strain and the original Peru-3 strain. One colony of the 25 was non-soft agar penetrating (when compared to the controls). This colony, designated Peru-14, was still Inaba positive with agglutination sera, and produced the
15 same level of B-subunit toxin as Peru-3 when tested in the B-subunit ELISA. The methods described above can be used for isolating soft agar penetration defective mutants of any *V. cholerae* strain. Non-revertable penetration-defective mutants, such as those harboring a
20 genetic deletion, can be made using the methods described above.

Bengal strains

A highly unusual non-01 virulent strain has recently been discovered to be responsible for a cholera
25 epidemic on the Indian sub-continent. Survivors of earlier 01 serogroup epidemics are not immunologically protected against this strain.

This strain has been deposited with the American Type Culture Collection (ATCC) in Rockville, MD. Bengal
30 can be attenuated as described above for the other strains, e.g., by one or more of the following mutations: *ctx*⁻, *att*⁻, or *recA*⁻, or a soft agar-defective phenotype, Bengal-2 ("Beng-2") and Bengal-3 ("Beng-3"), are genetically equivalent to Peru-2 and Peru-3. Such an
35 attenuated Bengal strain may be combined with one of the

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above-described attenuated Peru strains to provide a dual or multi-cholera strain vaccine.

Human testing of Peru-3 and Peru-5

Human studies of the efficacy of Peru-3 and Peru-5 were performed as follows.

Blood samples were drawn from volunteers prior to immunization and at 7, 14, 21, and 28 days post immunization. The *V. cholerae* antibodies in their blood stream were measured and levels are shown in Table 4.

10 The immunogenicity of vaccine prototypes Peru-3 and Peru-5 were evaluated in human volunteer studies. Volunteers ingested freshly harvested Peru-3 or Peru-5 at 3 different doses in 100ml of 10% sodium bicarbonate. Peru-3 and Peru-5 were also shown to induce antitoxin

15 antibodies (Table 5). In addition, Peru-3 and Peru-5 were shown to protect volunteers from challenge with a wild-type El Tor *V. cholerae* (strain N16961, Table 6). We conclude that Peru-3 in particular provokes a potent immune response (Tables 4 and 5) and confers protection

20 from cholera in human studies (Table 6).

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Table 4

Vibriocidal Titers After Immunization with Peru-3 or Peru-5 (July 1992)

	Strain(cfu)	Volunteer	Pre	7	14	21	28	Peak
5	Peru-3 (4×10^6)	1	50	1600	6400	6400	6400	6400
		2	<100	50	100	50	100	100
		5	<100	1600	6400	400	400	6400
10	Peru-3 (1×10^8)	7	<100	400	1600	400	400	1600
		12	<100	400	800	400	200	800
		13	<100	3200	6400	3200	1600	6400
15	Peru-5 (2×10^6)	11	<100	1600	6400	3200	6400	6400
		14	<100	200	6400	3200	1600	6400
		15	<100	800	6400	1600	3200	6400

Heat activated serum samples were serially diluted into microtiter wells, mixed with log phase *V. cholerae* (final concentration of 5×10^7) and guinea pig complement (final concentration of 11%) and incubated at 37°C for 1 hour.

20 Brain-Heart-Infusion broth was then added to plates and incubated at 37°C for 2.75 hours. Values in table represent the reciprocal titers at which antibody-mediated killing of *V. cholerae* was 50% or greater.

Table 5

25 Cholera Antitoxin Titers after immunization with Peru-3 or Peru-5 (July 1992)

	Strain(cfu)	Volunteer	.2	7	14	21	28	Peak Increase (fold)
30	Peru-3 (4×10^6)	1	8	8	32	32	32	4
		2	<2	<2	<2	<2	<2	None
		5	2	64	64	2	256	14
35	Peru-3 (4×10^8)	7	2	2	4	4	4	2
		12	<2	2	4	4	4	4
		13	<2	<2	<2	<2	<2	None
40	Peru-5 (2×10^6)	11	4	4	4	4	4	None
		14	2	2	2	2	2	None
		15	8	8	8	8	8	None

45 Serum samples were serially diluted into pre-treated, ganglioside/cholera toxin B-subunit coated 96 well microtiter plates and incubated at 37°C for 30 minutes. Following 3 washes with PBS, goat anti-human antibody-alkaline phosphatase conjugate (1/1000) was added and incubated at 37°C for 30 minutes. Following 3 washes with PBS, 2mg/ml PNPP was added to each well and incubated for 15 minutes. Reaction was stopped with 0.1M K_2PO_4 and read at an O.D. of 405nm. Values on the table represent the reciprocal titers and the increase of day-2 compared to peak titer.

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Table 6

Outcome for Volunteers Challenged with 2×10^6 cfu of Vibrio cholerae (N16961) wild-type Organisms (November 1992)

5	Subject Number	Previous Vaccination	Initial Dose	Symptoms	Diarrhea (grams)	Onset of Symptoms
	1	Peru-3	6 logs	None	Formed	
	2	Peru-3*	6 logs	Tired, gurgling	534	18-48 hours
10	5	Peru-3	6 logs	None	3	
	7	Peru-3	8 logs	None	23	36 hours
	11	Peru-5	6 logs	None	Formed	
	12	Peru-3	8 logs	None	Formed	
15	14	Peru-5	6 logs	None	Formed	
	15	Peru-5	6 logs	None	Formed	
	22	Control		T 100.7 F, HA, nausea LOA, gurgling, cramps	1443	24 hours
20	23	Control		None	769	24 hours
	24	Control		T 99.6 F, HA, malaise, gurgling, cramps	904+	40 hours
25						

- Did not colonize or subsequently seroconvert after vaccination
- + Two liquid stools not weighed due to urgency

Construction of *V. cholerae* vaccines expressing

30 heterologous antigens

The procedures described above can be applied by any artisan skilled in the art for the construction of derivatives of Peru-2, Bang-2, Bah-2, Peru-14, and related strains which are capable of expressing a wide
 35 variety of foreign or heterologous antigens, e.g., antigens that are not normally expressed in *V. cholerae*. Such derivatives, when used as live vaccines, would be expected to induce a strong immune response against both
V. cholerae antigens and the foreign antigen that it
 40 encodes. Both systemic and local immune responses will likely be induced because vaccination with other prototype *V. cholerae* vaccines has resulted in the induction of circulating IgG and local IgA antibodies

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that are specific for both whole cell antigens (e.g., LPS) and as well as individual proteins such as cholera toxin B subunit (Herrington et al., 1988, J. Exp. Med. 168:1487-1492). A foreign antigen expressed by *V.*

- 5 *cholerae* would be expected to elicit an immune response similar to that of the individual cholera proteins.

The methods useful for the introduction of heterologous antigens into *V. cholerae* are similar to those described above for the re-introduction of the *ctxB*
10 gene into vaccine strains Peru-3, Peru-4, Peru-14, Peru-5, Bang-3, Bah-3 and Bah-4. Virtually any heterologous antigen can be inserted into *V. cholerae* using these methods.

The same protocol used to construct *ctxB*
15 containing strains under a novel promoter can be used to construct derivatives of Peru-2, Bang-2 and Bah-2 which are capable of expression virtually any heterologous antigen or antigens normally encoded by either bacteria, viruses, or parasites. The methods described in the
20 invention therefore teach generation of a multivalent *V. cholerae* vaccine "carrier strain" which can be manipulated to encode and express other antigens and can be administered to humans in order to immunize them against not only cholera, but other pathogens as well.

25 *V. cholerae*/enterotoxigenic *E. coli* vaccines

Vibrio cholerae vaccines which elicit antibodies against cholera toxin (CT) have been demonstrated to confer cross protection to human vaccinees against strains of heat-labile toxin (LT) producing
30 enterotoxigenic *E. coli* (ETEC) (Svennerholm, J. Infect. Dis., 149:884-893, 1984). Vaccinees were still vulnerable however to heat-stable toxin (ST) producing strains of ETEC. An attenuated strain of *Vibrio cholerae*, Peru-3, can be used as a vaccine vector harboring ETEC-derived
35 foreign genes encoding the major subunit of colonization

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factor antigen CFA/IV fimbriae, and a genetic toxoid of ST. Such a vaccine vector will elicit i) anti-fimbrial antibodies, precluding binding of pathogenic ETEC strains to the human gut epithelium, and ii) anti-ST antibodies;
5 negating the diarrheal effects of ST. The result is a single dose orally administered live attenuated *V. cholerae* vectored ETEC vaccine.

The attenuated *V. cholerae* vectored ETEC vaccine may have one or more of the following advantages: i) it can
10 be lyophilized for long-term storage, ii) it requires no cold-chain, iii) it is orally administered, iv) it requires only a single-dose, v) it is cost effective, and vi) it protects against most ETEC strains.

A single-dose live oral vaccine directed against the
15 enteric pathogens, *V. cholerae* and enterotoxigenic *E. coli* (ETEC) is made by genetically engineering sequences encoding antigens from *E. coli* into the *V. cholerae* vaccine strains. In the construction of such vaccine strains, it is desirable to neutralize both colonization
20 and toxin production. This can be achieved by modifying an attenuated strain of *V. cholerae* , Peru-3 as described above.

Peru-3 strain already expresses cholera toxin B subunit which is nearly identical to the ETEC heat-labile
25 toxin B subunit, and elicits cross protective antibodies. The strain can be modified to express fimbrial antigens of ETEC and a chimeric protein made up of the oligomerization domain of cholera toxin A subunit and a mutant form of the ETEC heat-stable toxin. In this way,
30 the induction of immunity to both *V. cholerae* and *E. coli* can be accomplished.

The generation of the *V. cholerae*/ETEC vaccine strain is accomplished by utilizing common techniques in microbiology and molecular biology. The ability of the
35 strain to colonize animals and induce an immune response

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can be analyzed in an established model of enteric infection of rabbits.

Cloning and expression of fimbrial antigens

The ability of ETEC to colonize the intestinal
5 epithelium of humans is mediated by serologically
distinct fimbriae known as colonization factor antigens
(CFAs) and putative colonization factors (PCFs). The
CFA/4 fimbriae is the principal colonization factor
identified in approximately one quarter to one third of
10 all ETEC clinical isolates. The gene encoding the major
subunit of a prototype member of the group (CS6) has been
cloned and sequenced by others.

The cloned CS6 gene carried on a high-copy number
plasmid was introduced to Peru-3 via
15 electrotransformation and maintained by culture in Luria-
Bertani broth containing 50 µg/ml of ampicillin. Whole
cell lysates of Peru-3 containing the CS6 sequences were
analyzed for protein antigen expression by denaturing
polyacrylamide gel electrophoresis and immunoblotting
20 using anti-CS6 polyclonal rabbit serum. Immunoblots were
developed using anti-rabbit IgG-alkaline phosphatase
conjugate and BCIP. Expression of the CS6 gene was
detected as production of a 17-kiloDalton protein. Thus
CS6 antigen can be expressed in Peru-3 for the
25 formulation of a vaccine.

In order to generate the candidate vaccine strain,
however, it is desirable to have the CS6 gene stably
maintained in the absence of antibiotic selection and to
have it expressed from a promoter that is actively
30 transcribed by *V. cholerae*. To that end, the polymerase
chain reaction (PCR) can be used to specifically amplify
the CS6 gene carried on a plasmid and to create unique
restriction endonuclease sites at its termini for
subsequent cloning into an ampicillin resistant,
35 streptomycin sensitive "suicide" vector which allows

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integration onto the chromosome of *V. cholerae*. Specifically, PCR generated CS6 DNA flanked with a 5' *PacI* site and a 3' *NotI* site can be ligated with pJM6891 DNA which has been digested with *PacI* and *NotI*, placing
5 the CS6 gene under the control of the cholera toxin promoter. The ligation mixture can be introduced by electrotransformation into *E. coli* strain SM10pir which provides a specific trans-acting protein, known as *pi*, required by pJM6891 for replication. However, when
10 pJM6891 and its derivatives are introduced into *V. cholerae* (which lacks the *pi* protein), selection for resistance to 50 µg/ml of ampicillin requires that the plasmid integrate onto the chromosome. The site of integration is determined by the presence of *V. cholerae*
15 *lacZ* DNA sequences flanking CS6 which are identical to sequences on the *V. cholerae* chromosome and allow homologous recombination to occur. The resulting progeny is ampicillin resistant and harbors an integrated copy of the plasmid and CS6 sequences surrounded by repeated DNA
20 sequences of the *lacZ* gene.

The repeats can be resolved to remove the vector sequences (including the ampicillin resistance determinant), leaving the CS6 gene under control of the toxin promoter. This is performed by culturing the
25 strain in the presence of 2 mg/ml of streptomycin, selecting for the streptomycin resistance allele native to Peru-3 and against the streptomycin sensitivity allele introduced by the plasmid. After growth overnight in the presence of streptomycin, the culture is plated
30 for single colonies on LB agar containing 100 µg/ml streptomycin, and scored for sensitivity to 50 µg/ml ampicillin. Isolates that are streptomycin sensitive and ampicillin resistant will be analyzed by Southern blots of chromosomal DNA to determine if the expected
35 integration and excision events have occurred.

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These isolates can be analyzed for level of antigen production using immunoblotting techniques. Production of CS6 fimbrial antigen can be evaluated under a variety of growth conditions known to affect transcription of the cholera toxin promoter. The effect of media pH (6.5 versus 8.0), temperature (30°C versus 37°C), NaCl concentration (50 to 500 mM) and amino acid concentration (0 to 25 mM) on the level of CS6 expression can be determined.

10 The candidate vaccine strain Peru-3/CS6 can then be used in a rabbit model to demonstrate safety and immunogenicity. Since the human clinical isolates of ETEC that produce CFA antigens are typically not pathogenic to laboratory animals, another Peru-3
15 derivative expressing the AF/R1 fimbrial antigen of the *E. coli* strain RDEC-1 can be constructed in order to demonstrate safety and immunogenicity. This antigen mediates adherence to gut epithelium, causing a diarrheal disease in rabbits. The gene encoding AF/R1, carried on
20 the plasmid pW1, can be amplified by PCR, cloned into pJM6891 and integrated into the chromosome in the same manner as for CS6. The level of AF/R1 expression can be evaluated by immunoblotting. While this will not produce a vaccine candidates for humans, it can serve as a model
25 for demonstrating the expression of heterologous antigen by modified Peru-3 strains and the induction of protection from challenge by a heterologous organism.

The cloned AF/R1 gene carried on a high-copy number plasmid was also introduced to Peru-3 via
30 electrotransformation and maintained by culture in Luria-Bertani broth containing 50 µg/ml of ampicillin. Whole cell lysates of Peru-3 containing the AF/R1 sequences were analyzed for protein antigen expression by denaturing polyacrylamide gel electrophoresis and
35 immunoblotting using anti-AF/R1 polyclonal rabbit serum.

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Immunoblots were developed using anti-rabbit IgG-alkaline phosphatase conjugate and BCIP. Expression of the AF/R1 gene was detected as production of approximately 18-kiloDalton protein. Thus AF/R1 antigen can be expressed
5 in Peru-3 for the formulation of a vaccine.

Using similar strategies, a vaccine strain expressing protective antigens of *Shigella*, such as lipopolysaccharide (LPS) and plasmid-derived invasive protein, can be made to protect against infectious
10 diarrhea caused by infective species of *Shigella*, such as *S. sonnei*.

In *S. sonnei*, there is only one serotype of LPS and it is the primary antigenic determinant in protection against this bacteria. Introduction of a plasmid clone
15 encoding the LPS operon into *E. coli* results in expression of LPS and is sufficient to confer upon *E. coli* the ability to be agglutinated by anti-*S. sonnei* LPS antibodies. The same plasmid introduced into the Peru-3 deletion mutant strain renders it agglutinatable.
20 Further analysis of the operon indicated that a 12 kilobase EcoR1/BamH1 fragment of this plasmid subcloned into pBR322 still confers the agglutination phenotype. This fragment can then be introduced to the chromosome at the *lacZ* gene of *V. cholerae* as described above.

25 Construction and safety of ST-CTA2 fusions.

ETEC causes diarrhea by colonization and production of two distinct toxins. The heat-labile toxin (LT) is nearly identical in sequence, structure and biological action to cholera toxin (CT). Therefore, production of
30 CT by Peru-3 derivatives is sufficient to induce antibodies capable of neutralizing both toxins. However, immunization with CT cannot confer protection from the ETEC heat-stable toxin (ST) which is a very small (19 amino acids) polypeptide produced by many clinical
35 isolates, some of which do not produce LT. Thus a

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critical element in the candidate cholera/ETEC vaccine is the inclusion of ST sequences in Peru-3 in order to induce antibodies to this toxin.

A number of well defined derivatives of ST have been generated that are devoid of toxin activity (SToxoids). These derivatives are typically fragments of the toxin or substitution mutations in cysteine residues that form the three disulfide bonds of the protein. An SToxoid made up of the entire mature polypeptide with cysteine to alanine mutations in residues 5 and 10 can be constructed to minimize or eliminate toxic activity. The gene encoding this SToxoid can be made entirely from complementary oligonucleotides produced with a DNA synthesizer. The synthetic gene can be flanked by unique restriction endonuclease sites for subsequent subcloning into plasmid vectors.

The size of ST (19 amino acids) renders it an inherently poor immunogen. If intact ST or even small peptide fragments are coupled chemically or genetically to other larger proteins (a carrier), ST becomes a much better immunogen and can induce neutralizing antibodies. The principal carrier used was the B subunit of LT or CT. Since foreign proteins fused to the cholera toxin A2 subunit (the domain of the enzymatic subunit which allows the A fragment to oligomerize with the B subunit pentamer) can bind to the pentamer and form holotoxin-like complexes, these chimeric complexes are i) secreted by *V. cholerae*, ii) capable of binding the ganglioside receptor, and iii) immunoreactive.

The synthetic gene encoding SToxoid can be fused, in frame, to the 5' end of the gene encoding CT A2 creating an SToxoid-A2 chimera. The gene fusion construct can be integrated onto the Peru-3 chromosome as described above. When co-expressed with CT B subunit, this protein can form holotoxin-like complexes devoid of both ST and CT

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biological activity and capable of binding the ganglioside receptors. Strains expressing the SToxoid-A2 chimeric protein can be analyzed by immunoblots using anti-ST antiserum to determine if the substitution
5 mutations result in an antigenically related protein. The SToxoids can also be compared for toxicity in the infant mouse assay.

The infant mouse assay is carried out as follows. 2-3 day old mice are injected intragastrically with
10 protein extracts derived from these vaccine strains (or purified ST as a control), sacrificed 3-4 hours after injection and examined for increased gut-to-body weight ratio. Candidate SToxoid-A2 chimeras demonstrating the lowest toxicity, can then be analyzed for immunogenicity
15 in rabbits.

Safety, immunogenicity and efficacy of Peru-3/AF/R1.

Initial testing of the Peru-3 expressing AF/R1 can be done in rabbits. Bacteria can be administered orally at doses of 2×10^2 , 2×10^4 , 2×10^6 , and 2×10^8 to New
20 Zealand White rabbits. Stool samples can be collected and cultured on LB agar plates with 100 $\mu\text{g/ml}$ streptomycin to enumerate colonization and shedding of bacteria. Blood can be drawn before administration of the vaccine as well as 7, 14, 21 and 28 days following
25 administration. Sera can be prepared and analyzed for the presence of antibodies specific for AF/R1 protein via an enzyme-linked immunosorbant assay (ELISA) using purified AF/R1 bound to microtiter plates, and ability to agglutinate RDEC-1 bacteria.

30 Animals receiving the Peru-3/AF/R1 strain can be subsequently challenged with a pathogenic strain of RDEC-1. A challenge dose of 2×10^6 organisms can be administered orally to immunized and naive rabbits and stool samples observed for diarrhea (defined as loose,
35 wet stool soiling the rectal area and loose stool in the

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cage bottom). Diarrhea typically occurs within 3-4 days in non-immune animals. To assay colonization, rectal swab samples are cultured on lactose MacConkey agar plates and lactose positive colonies are scored for positive reaction with anti-RDEC-1 antibodies in a slide agglutination test. Protection can be defined as both inhibition of diarrhea and bacterial colonization after day four.

Safety and immunogenicity of Peru-3/CS6 and Peru-3/SToxoid. Initial testing of the Peru-3 strains expressing CS6 and SToxoid-A2 can be done as described above. Sera can be prepared and analyzed for the presence of antibodies specific for either CS6 or SToxoid-A2 chimeric protein via an enzyme-linked immunosorbant assay (ELISA) using purified CS6 or ganglioside bound to microtiter plates. The anti-CS6 sera can also be analyzed for the presence of antibodies capable of fixing complement and lysing CS6 producing *E. coli*. In this assay, bacteria bearing CS6 are mixed with serum and guinea pig complement, LB broth is added, and the bacteria are plated on LB agar. Bacteriocidal activity results in a decrease in the viable counts recovered. Finally, the anti-SToxoid-A2 sera can be tested for antibodies capable of neutralizing ST activity in the infant mouse toxicity assay.

Construction of attenuated *Vibrio.cholera* expressing HIV-1 antigen as recombinant cholera holotoxoid

An approach similar to that described above can be used to construct a *V. cholerae* vaccine strain expressing antigens of the Human Immunodeficiency Virus (HIV).

A cholera shuttle plasmid which contains a bacterial transcription unit including the promoter of the heat shock protein, *htp*, and the cholera CT-B gene was constructed. The transcription unit is flanked by the DNA sequences derived from the *recA* locus of *cholera* so

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that the CT-B gene and its promoter can be integrated into the *cholera* chromosome by the homologous recombination between the DNA sequence presence both in the *recA* locus of the *cholera* genome and on the plasmid.

- 5 The shuttle plasmid also contains a gene encoding ampicillin resistance and a gene encoding streptomycin sensitivity as the selection markers.

The HIV-1 envelope protein can be expressed as a part of recombinant *V. cholerae* holotoxoid secreted by
10 the bacteria, in the form of a "sandwich" fusion protein, in which the HIV antigen is preceded by the signal sequences of the CT-A polypeptide and followed by the CT-A2 domain. The signal sequences of the CT-A and its upstream untranslated region are required for the
15 expression and secretion of the HIV-1 antigen in the bacteria. The CT-A2 domain fused to the HIV-1 antigen is required for the fusion protein to assemble with the CT-B proteins to form a recombinant cholera holotoxoid. The plasmid described above can be modified such that a PCR
20 fragment containing the Shine-Dalgarno (SD) sequences and the signal sequences of the CT-A gene, and a unique restriction endonuclease *PmeI* site for inserting the HIV-1 antigen is inserted into its *PacI* site. The plasmid can further be modified such that a second PCR fragment
25 containing both the CT-A2 domain and the CT-B gene replaces the CT-B gene. The orientation of the DNA insertion and the junction of the PCR fragment can be confirmed by DNA sequencing.

The HIV-1 antigen used in this study is a part of
30 the HIV-1 envelope glycoprotein containing the principle neutralizing domain (PND). Previous studies demonstrate that a group of synthetic peptides derived from the PND can elicit neutralizing antibody in animals. A DNA fragment derived from HIV-LAI envelope gene including the
35 PND, but without the signal sequences and the first 120

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amino acids, is cloned into the PmeI site of the plasmid described above which contains both the CT-A2 domain and the CT-B gene. The *in frame* fusion of HIV-1 antigen and the CT-A signal peptide and CT-A2 domain can be confirmed
5 by DNA sequencing.

To construct a genetically attenuated *cholera* strain that carries the HIV-1 antigen, Peru-2 is used the parental strain. The plasmid containing HIV sequences can be introduced into Peru-2 strain by mating. A
10 recombinant strain of *V.cholera* which contains deletions of *ctx* and *recA* loci and expresses a non-toxic recombinant fusion protein of HIV-1 antigen was produced and named Peru101. Southern Blot analysis can be used to confirm that Peru101 contains the DNA for HIV-1 antigen
15 and Western blot analysis can be used to demonstrate the expression of HIV-A2 fusion protein by the recombinant bacteria. An ELISA using both anti-CT-B and anti-HIV antibodies can test if the recombinant *cholera* holotoxoid is secreted by the bacteria.

20 Preclinical evaluation in primates of immunogenicity and protective efficacy of the oral HIV 1 vaccines using SHIV model

To test the immunogenicity of *V.cholerae* recombinant Peru101 as an oral HIV-1 prophylactic vaccine, each of
25 six adult female Rhesus monkeys (*Macaca mulatta*) can be given 2×10^6 CFU freshly prepared live bacteria in 30 ml bicarbonated water. Two additional animals in the same age and sex group can be given the same dose of Peru 2 as a control. The stool samples of the animals can be
30 analyzed two days after the vaccination to detect the multiplication of *Vibrio cholera* in the intestines by determining the colony forming unit on the LB streptomycin plates. The vaginal, rectal, salivary and serum antibodies, including IgA and IgG, that are
35 specific to HIV1 and to the CT-B can be examined biweekly

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post vaccination. The host animal's T cell proliferation and CTL responses that are specific to the input HIV1 antigen can also be examined. One or several boosts by oral, or by intramuscular and
5 intravenous injection of purified HIV1 antigen may be necessary, depending upon the level of the initial immune responses of the vaccinated animals.

If Peru101 is able to stimulate the animals to generate anti-HIV antibodies or cell mediated HIV1
10 specific immune responses, the efficacy of Peru101 as HIV1 vaccine can be tested by challenging the animals with live SHIV-LAI stocks through vaginal infusion. The two Peru 2 animals (the monkeys who received Peru2 strain) and two of the six Peru101 animals (the monkeys
15 who received Peru101) can be challenged by 2x VI-AID₅₀ dose. Two of the other Peru101 animals will receive 10x VI-AID₅₀ and the rest of the Peru101 monkeys will receive a maximum of 50x VI-AID₅₀ dose. The peripheral blood samples can be collected every two weeks post infection
20 to determine if the animal becomes infected by detecting the viral antigen in the cultured PBMC. If the vaccine has prophylactic effect on the animals against the challenge by the SHIV carrying homologous HIV1 envelope gene, SHIV-Eli, which contains a heterologous HIV1
25 envelope, can be used to re-challenge the animals.

Use of the Live Vaccine Strains

The *V. cholerae* mutant strains Peru-1, Peru-2, Bang-1, Bang-2, Bah-1, Bah-2, Bengal -2, Bengal -3, Peru-14, and the additional mutants described above are useful as
30 sources of immunological protection against cholera and other related toxigenic diseases when used as live vaccines. Other such diseases include, but are not limited to, those induced by enterotoxigenic *E. coli* and other bacteria that produce toxins which are

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immunologically cross-neutralizable with cholera B subunit.

When inoculated into the intestine of an experimental animal or human, mutant strains of *V. cholerae* should stimulate and induce a strong immunological response against all bacterial components that are elaborated by these strains including, but not limited to, the Ogawa and Inaba 01 LPS antigens, flagella antigens, the antigenic domains of the Tcp pili, and the outer membrane proteins. Based on published studies with other prototype cholera vaccines, both IgA and IgG classes of antibodies directed against these bacterial components will be synthesized in the inoculated animal or human and will serve to protect the animal or human against subsequent challenge with virulent strains of *V. cholerae*.

Dosage

Determination of the appropriate dosage and administration of these vaccines is performed essentially as described in Herrington et al., (1988, J. Exper. Med. 168:1487-1492). In general, such dosages are between, but are not limited to, 10^5 - 10^9 viable bacteria per dose.

Growth of Vaccine Strains

The bacteria to be used as the vaccine can be grown in a standard *V. Cholerae* laboratory media. The cells can be harvested and then lyophilized in a formulation that preserves viability (e.g., sterile skim milk or saline containing 5mM CaCl_2 and 10% weight by volume of glycerol).

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Administration

Administration of the vaccine involves combining the contents of two envelopes or vials, one containing the lyophilized vaccine strain or combination of strains, the other containing water and sufficient sodium bicarbonate or alternate buffer as to neutralize stomach acid (approximately 2 grams). The vaccine can then be swallowed by the vaccinee. Alternatively, the lyophilized vaccine can be incorporated into tablets which can be coated with an acid resistant "enteric coating". Such a form of vaccine can be administered to the vaccinee in one or more (up to three) doses spaced from a few days to several weeks apart. When used as a "booster" vaccine, the vaccine can also be administered to previously vaccinated individuals in one or more doses (up to three) spaced from a few days to several weeks apart. When two or more strains are being administered they may be provided together, or in individual doses 7-28 days apart.

Improved Killed Oral Cholera Vaccines

Preparations of improved killed oral cholera vaccines can be made from the strains described above. The experimental cholera vaccine that is currently available is comprised of approximately 10^{11} formalin and heat killed *V. cholerae* cells mixed with purified cholera toxin B subunit (Black et al., Infect. Immun. 55:1116, 1987). The four strains that are used in the preparation of the bacterial component of this vaccine produce active cholera toxin which must be completely inactivated before administration to the vaccinee. The new strains described above provide a vaccine that is vastly improved compared with the vaccine of Black et al. (Supra) for each of the reasons given below.

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(1) Because the strains derived from, and including Peru-2, Bang-2 and Bah-2, produce only the nontoxic B subunit of the cholera toxin and not the toxic A subunit, cultures of these strains require only mild inactivation 5 prior to administration to a vaccinee, thus avoiding the more severe denaturing treatments such as formalin or heat. The advantages of the milder treatment are that the antigens will retain a greater degree of their native configuration and as a result they will be more 10 immunogenic. Mild methods of inactivation that avoid chemically inactivating the bacterial proteins include microwaving the organisms, treatment with another radiation source or a mild organic solvent or detergent, or the cells may be lysed by mechanical methods such as 15 sonication or use of a French Press.

(2) In the strains Peru-3, Bang-3 and Bah-3, the *ctxB* gene has been placed under the control of the *htp* promoter. As a result, these strains synthesize large quantities of the cholera toxin B subunit (greater than 20 10 µg/ml of culture) in standard laboratory medium such as LB. This facilitates purification of large amounts of the cholera B subunit and thus these strains provide a significant advantage over other strains which only produce the B subunit in small quantities under stringent 25 growth conditions.

(3) In the preparation of existing killed cholera vaccines, a separate bacterial strain is used to produce the B toxin subunit from the strain used as the whole cell antigen. During preparation of the B subunit it is 30 therefore necessary to purify the B subunit away from the toxic A subunit using biochemical methods. Such purification incurs the risk that small amounts of the A subunit may contaminate the preparation of the B subunit. Using the strains described above, it is possible to 35 generate a whole cell antigen preparation from the same

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culture used to obtain the B subunit preparation. In the first instance, purification of the B subunit is now unnecessary because the strain does not produce the A subunit, thus reducing the amount of time and
5 considerable expense involved in production of the vaccine. Secondly, there is no risk of having any contaminating A subunit in the preparations since the bacteria simply do not encode the gene for this subunit and therefore cannot produce it. The whole cell
10 preparation can therefore be used as a vaccine with minimal risk to the vaccinee.

(4) Some bacterial strains of the invention are derivatives of *V. cholerae* of the El Tor biotype and more particularly, in the case of Peru-2, Peru-3 and Peru-4,
15 they are derivatives of an isolate (C6709-Sm) which is in fact the causative agent of the current epidemic in Latin America. If there are antigens that are unique to this particular parental strain, the vaccine derivatives described above may provide generally better protection
20 against El Tor disease in Latin America and possibly other areas in the world.

Preparation of Improved Oral Killed Cholera Vaccines

An improved oral killed cholera vaccine can be prepared as follows. A minimum of two strains,
25 preferably, selected from the Ogawa serotype (e.g., Bah-3), to the Inaba serotype (e.g., Peru-3, Peru-14, or Bang-3), and the Bengal serotype (e.g., Bengal-2 or Bengal-3) can be grown in separate cultures. One of ordinary skill in the art will know how to adjust the
30 conditions, media, etc. to maximize cell growth at 37°C. For example, cultures grown under a high level of aeration in a medium such as CYE (Mekalanos et al., 1977, Infect. Immun. 16:789) or minimal medium containing glucose, i.e., AGM4 (van de Walle et al., 1990, Appl.
35 Microbiol. Biotechnol. 33:389) can be used. When growth

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of the bacteria has reached saturation, whole cells can be recovered from the medium by centrifugation, while proteins (including the B subunit) contained within the supernatant fraction can be obtained by

5 ultracentrifugation or by precipitation. The cells can be inactivated using the methods of Black et al. (Infect. Immun. 55:1116, 1987) or by milder methods (e.g., microwaving, irradiation using alpha, beta or gamma

10 rays), treatment with organic solvents such as ethanol or acetone, or they may be lysed by treatment with either a detergent or by mechanical methods, such as sonication or by using a French Press. The inactivated cells can then be combined with filtered, concentrated supernatant containing bacterial proteins (including subunit B) and

15 the mixture can be suspended in a pharmaceutically acceptable solution appropriate for oral administration (e.g., sterile saline or 2% sodium bicarbonate).

Administration The vaccine can be administered to the vaccinee as an oral saline solution which is swallowed by

20 the vaccinee several minutes after the vaccinee has ingested 2 grams of sodium bicarbonate. Alternatively, the preparation can be lyophilized and compressed into tablets which are then coated with an acid-resistant "enteric coating" prior to administration to the

25 vaccinee. The tablets can also be microencapsulated with polymers in order to facilitate uptake of the preparation by the intestinal mucosal tissue.

Dosage A single dose of vaccine should contain approximately 10^{11} cells and approximately 100-5000 μg of

30 cholera B subunit. It is expected that the vaccinee will require approximately two or more separate doses of vaccine administered approximately two or more weeks apart.

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Deposit

Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, 5 deposit of *V. cholerae* strains C6709-Sm, P27459-Sm, E7946-Sm, Bengal-2, Bengal-3, MO10, and Peru-14 have been made with the American Type Culture Collection (ATCC) of Rockville, Maryland, USA, where the deposits were given ATCC Accession Numbers ATCC 55331 (C6709-Sm); ATCC 55333 10 (P27459-Sm); ATCC 55332 (E7946-Sm); ATCC 55436 (0139, Bengal-2; ATCC 55437 (0139, Bengal-3); and ATCC 55438 (0139, MO10). Peru-14 was deposited with the ATCC June 30, 1993.

Applicant's assignee, President and Fellows of 15 Harvard College, represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably 20 removed upon granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 C.F.R. 1.14 and 35 U.S.C. § 122. The deposited material will be maintained with all the care 25 necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited material, and in any case, for a period of at least thirty (30) years after the date of deposit or for the 30 enforceable life of the patent, whichever period is longer. Applicant's assignee acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

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SEQUENCE LISTING**(1) GENERAL INFORMATION:**

- (i) APPLICANT: Mekalanos, John J.
- (ii) TITLE OF INVENTION: DELETION MUTANTS AS VACCINES FOR CHOLERA
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Fish & Richardson
 - (B) STREET: 225 Franklin Street
 - (C) CITY: Boston
 - (D) STATE: Massachusetts
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
 - (B) COMPUTER: IBM PS/2 Model 50Z or 55SX
 - (C) OPERATING SYSTEM: MS-DOS (Version 5.0)
 - (D) SOFTWARE: WordPerfect (Version 5.1)
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Freeman, John W.
 - (B) REGISTRATION NUMBER: 29,066
 - (C) REFERENCE/DOCKET NUMBER: 00742/007001
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (617) 542-5070
 - (B) TELEFAX: (617) 542-8906
 - (C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:**(i) SEQUENCE CHARACTERISTICS:**

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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17

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- 53 -

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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- (A) LENGTH: 32
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

SUBSTITUTE SHEET

- 54 -

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32

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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26

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What is claimed is:

1. A nontoxinogenic genetically stable mutant strain of *V. cholerae*, said strain being a genetically engineered deletion mutant lacking DNA encoding a functional *ctxA* subunit, said mutant further lacking any functional *attRS1* sequences.
2. A method of making a genetically stable mutant strain of *V. cholerae* lacking DNA encoding a functional *ctxA* subunit and further lacking any functional *attRS1* sequences, said method comprising introducing into a wild type *V. cholerae* a plasmid comprising a fragment of *V. cholerae* DNA which is mutated in its *ctxA* and *attRS1* sequences, said DNA being capable of recombining with wild type *V. cholerae* DNA inside said *V. cholerae* resulting in the generation of said mutant strain.
3. The *V. cholerae* strain of claim 1, wherein said strain is derived from a parental strain belonging to the El Tor serogroup.
4. The *V. cholerae* strain of claim 3, wherein said strain is derived from a parental strain belonging to the Inaba or Ogawa serotype.
5. The *V. cholerae* strain of claim 4, wherein said strain is Peru-2, Bang-2 or Bah-2.
6. The *V. cholerae* strain of claim 1, wherein said strain is derived from the non-O1 serogroup.

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7. The *V. cholerae* strain of claim 1, wherein said strain lacks CTX core sequences and is fully deleted for all said attRS1 sequences.

8. The *V. cholerae* strain of claim 1, wherein
5 said strain further lacks a functional *recA* gene.

9. The *V. cholerae* strain of claim 1, 7 or 8, wherein said strain further encodes a B subunit of cholera toxin.

10. The *V. cholerae* strain of claim 1, 7 or 8,
10 wherein said strain further encodes a heterologous antigen.

11. The *V. cholerae* strain of claim 10, wherein said heterologous antigen is a Shiga-like toxin or a *Shigella* lipopolysaccharide antigen, or an *E. coli*
15 fimbrial antigen or an HIV antigen.

12. The *V. cholerae* strain of claim 10, wherein the DNA sequence encoding said heterologous antigen is inserted into the *lacZ* gene of *V. cholerae*.

13. The *V. cholerae* strain of claim 9, wherein
20 said strain is Peru-3, Peru-4, Bang-3, Bah-3 or Bah-4.

14. The method of claim 2, wherein said *V. cholerae* strain is Peru-2, Bang-2 or Bah-2.

15. The method of claim 2, wherein said mutant strain lacks CTX core sequences and all attRS1 sequences.

25 16. The method of claim 2, wherein said mutant strain further lacks a functional *recA* gene.

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17. The method of claim 2, wherein said mutant strain further encodes a heterologous antigen.

18. The method of claim 2, wherein said method further comprises introducing into the *lacZ* gene of said
5 mutant strain a fragment of DNA encoding an antigen.

19. The method of claim 18, wherein said mutant strain is Peru-5, Bang-5 or Bah-5.

20. A killed oral cholera vaccine, said vaccine comprising at least a first and a second *V. cholerae* strain suspended in a physiologically acceptable carrier, wherein each strain lacks DNA encoding a functional *ctxA* subunit, and wherein at least two of said strains are different serotypes, said *V. cholerae* being non-viable, said vaccine further comprising cholera toxin
10 B subunit which is overproduced by at least one of said serotypes of said *V. cholerae* strain.
15

21. The vaccine of claim 20, wherein one of said serotypes is an Ogawa serotype and another of said serotypes is an Inaba serotype.

20 22. The vaccine of claim 21, wherein said vaccine comprises Bah-3 and either Peru-3 or Bang-3 or both Peru-3 and Bang-3.

23. A nontoxinogenic genetically stable mutant strain of *V. cholerae*, said strain being a genetically
25 engineered deletion mutant lacking DNA encoding a functional *ctxA* subunit, said strain being a soft agar penetration-defective mutant.

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24. A vaccine comprising at least two different strains of *V. cholerae* according to claim 23, one of said strains being derived from Peru and the other being derived from Bengal.

5 25. The vaccine of claim 24, wherein each of said strains is ctx⁻, att⁻, and recA⁻.

26. The vaccine of claim 23 wherein said strain is att⁻.

27. A method of making a killed *V. cholerae*
10 vaccine, said method comprising the steps of
 providing at least the first and second *V. cholerae* strains of claim 20, which strains have been killed;

 adding to said killed strains cholera toxin B
15 subunit produced by at least one of said strains, wherein said toxin B subunit is obtained from the medium in which said strain was propagated; and

 suspending said killed strains and said toxin B subunit in a physiologically acceptable carrier.

20 28. A vaccine comprising the strain of claim 1 in a physiologically acceptable carrier.

29. The *V. cholerae* strain of claim 4, wherein said strain is Peru-14.

30. The *V. cholerae* strain of claim 6, wherein
25 said strain is of the Bengal serogroup.

31. The *V. cholerae* strain of claim 6, wherein said strain is Bengal-2 or Bengal-3.

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32. The *V. cholerae* strain of claim 23, wherein at least 25% of the cells of said strain are capable of forming fillamentous structures of 15nM or greater under conditions of stationary phase growth.

CTX GENETIC ELEMENT

 = attRS1

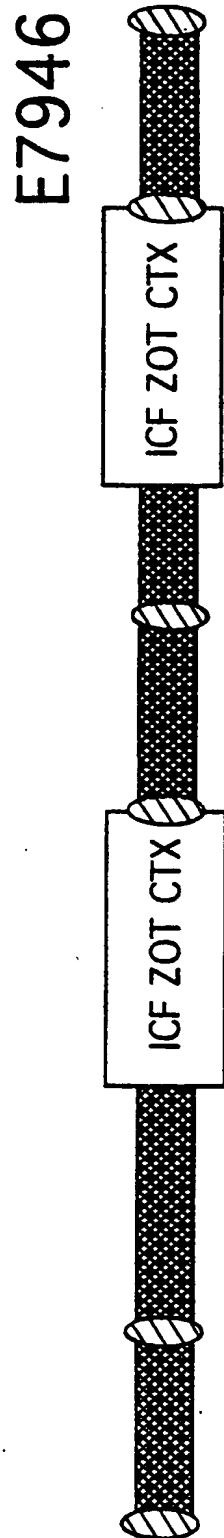
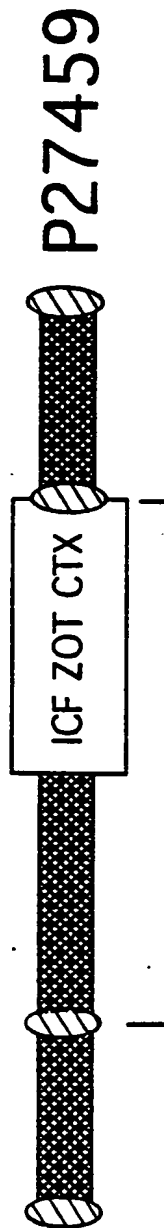
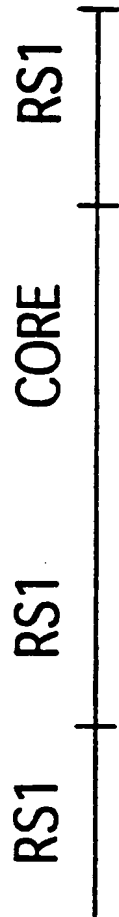


FIG. 1

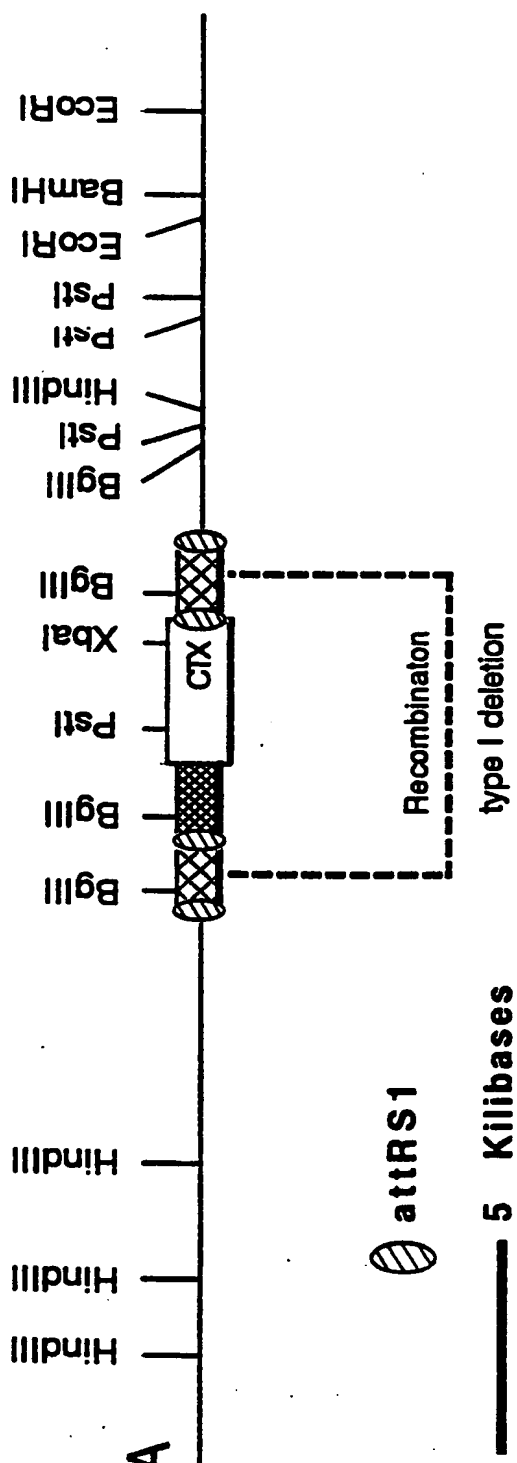


FIG. 2A

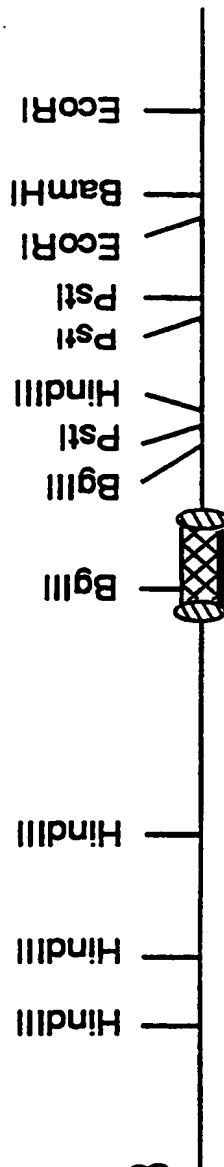
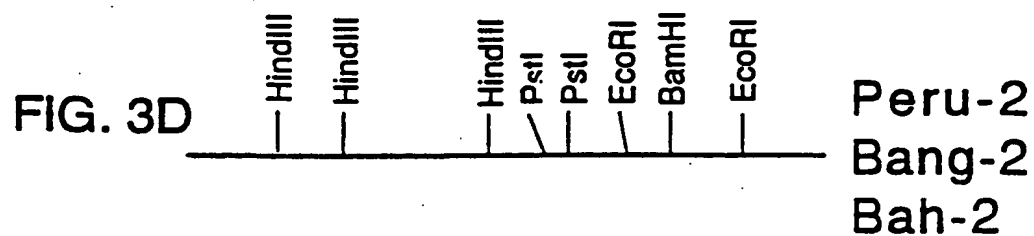
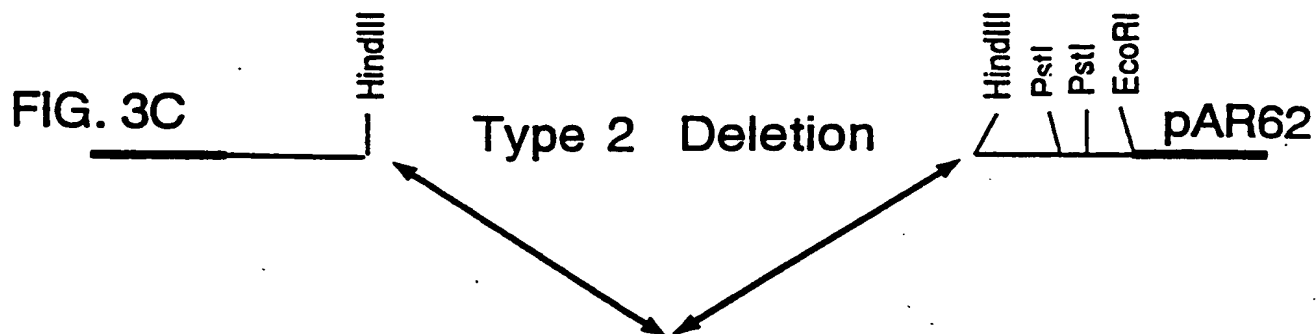
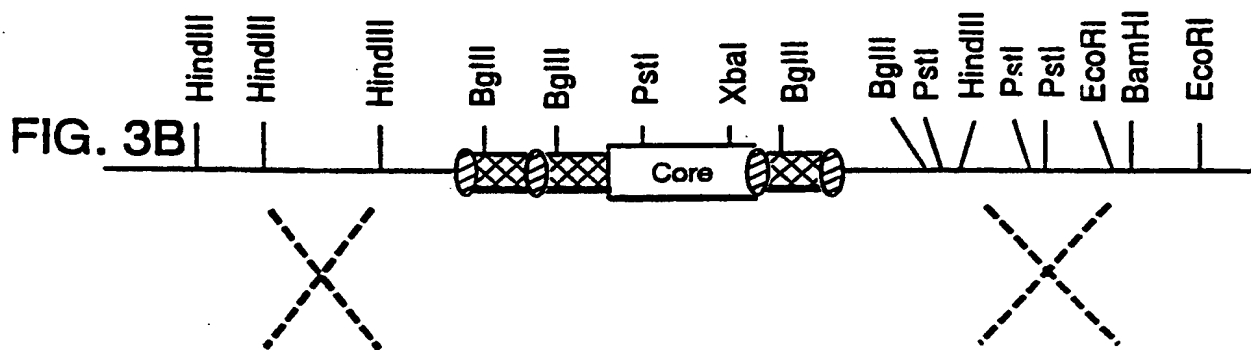
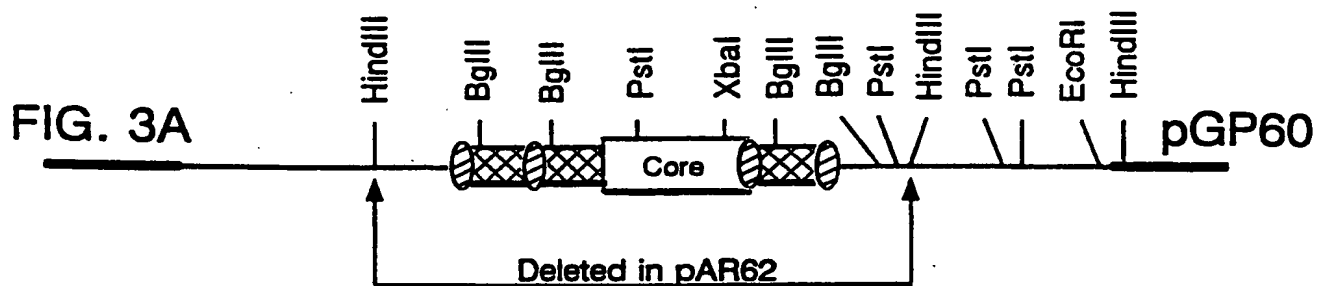


FIG. 2B

3/6

 = attRS1 sites



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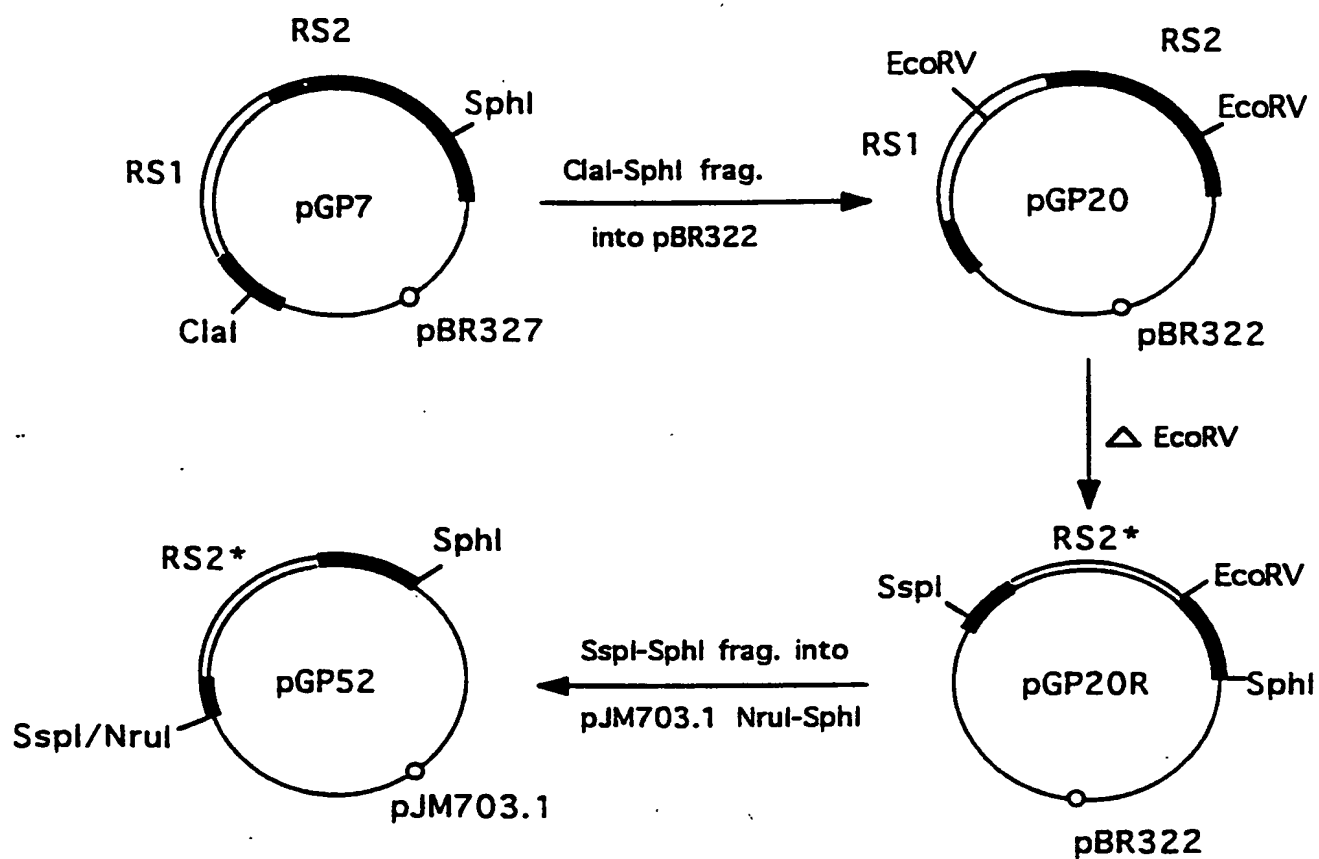


FIG. 4

5/6

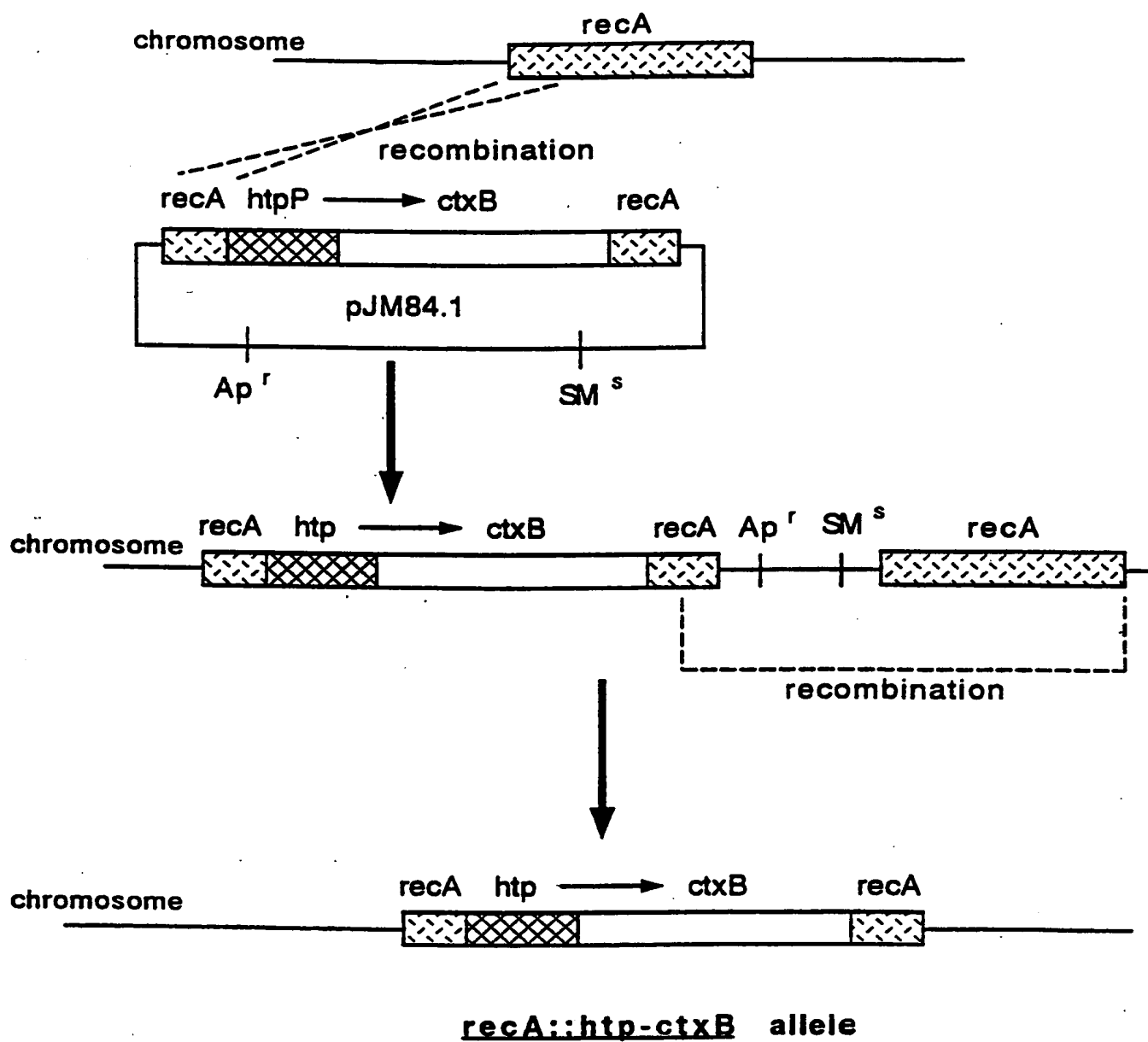
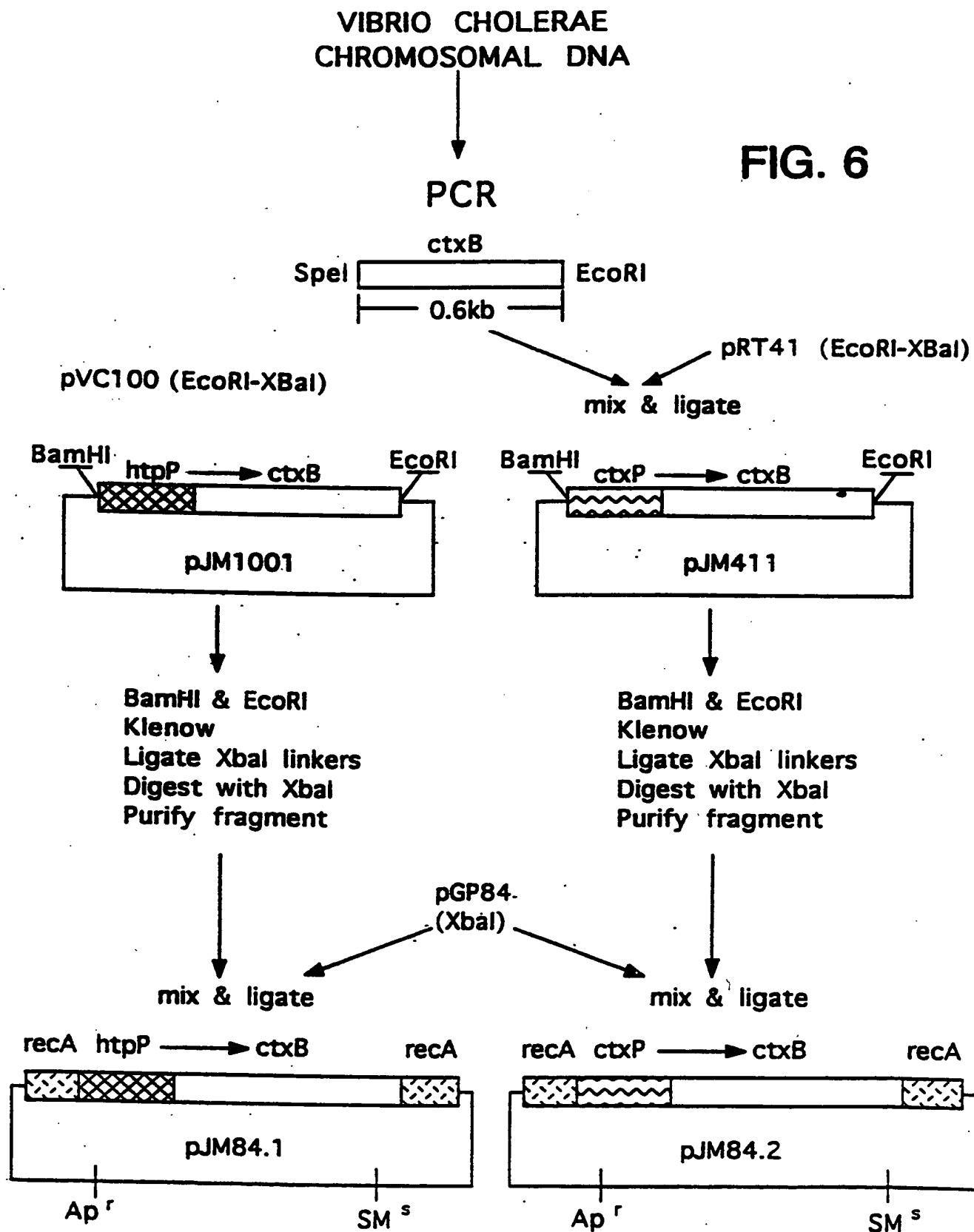


FIG. 5

SUBSTITUTE SHEET

6/6

FIG. 6



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.

PC /US93/06270

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 1/21; A61K 39/106

US CL :424/92, 93A; 435/172.1, 172.3, 909; 935/38, 65

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/92, 93A; 435/172.1, 172.3, 909; 935/38, 65

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,882,278 (MEKALANOS) 21 November 1989, see entire document.	1-32
Y	RESEARCH IN MICROBIOLOGY, Volume 141, issued 1990, G.D.N. Pearson et al, "New Attenuated Derivatives of <u>Vibrio cholerae</u> ", pages 893-899, see entire document.	1-19, 23-26, 28-32
Y	A.I. LASKIN et al, "CRITICAL REVIEWS IN MICROBIOLOGY", published May 1973, see pages 533-623.	5, 13, 14, 19, 20-22, 27

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, Volume 35, Number 1, issued November 1983, J.J. Mekalanos et al, "Duplication and Amplification of Toxin Genes in <u>Vibrio cholerae</u> ", pages 253-263, see entire document.	1-19, 23-26, 28-32
Y	THE LANCET, Volume 337, Number 8749, issued 04 May 1991, I.K. Wachsmuth et al, "Difference between Toxigenic <u>Vibrio cholerae</u> 01 from South America and US Gulf Coast", pages 1097-1098, see entire document.	20-22, 27
Y	BIO/TECHNOLOGY, Volume 2, issued April 1984, J.B. Kaper et al, "A Recombinant Live Oral Cholera Vaccine", pages 345-349, see entire document.	1-19, 23-26, 28-32
Y	DISSERTATION ABSTRACTS INTERNATIONAL, Volume 52, issued April 1992, G.D.N. Pearson et al, "The Cholera Toxic Element: A Site-Specific Transposon", see page 5094.	1-19, 23-26, 28-32
Y	APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, Volume 33, Number 4, issued July 1990, M. van de Walle et al, "Production of Cholera Toxin Subunit B by a Mutant Strain of <u>Vibrio cholerae</u> ", pages 389-394, see entire document.	20-22, 27
Y	JOURNAL OF CLINICAL MICROBIOLOGY, Volume 26, Number 10, issued October 1988, T. Yamamoto et al, " <u>Vibrio cholerae</u> Non-01: Production of Cell- Associated Hemagglutinins and In Vitro Adherence to Mucus Coat and Epithelial Surfaces of the Villi and Lymphoid Follicles of Human Small Intestines Treated with Formalin", pages 2018-2024, see abstract.	6, 30, 31
Y	JOURNAL OF GENERAL MICROBIOLOGY, Volume 137, Number 12, issued December 1991, W.T. Wibawan et al, "Influence of Capsular Neuramic Acid on Properties of Streptococci of Serological Group B", pages 2721-2725, see entire document.	23-26, 32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06270

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, HEALTH PERIODICALS, DISSERTATION ABSTRACTS, MEDLINE, LIFE SCIENCE COLLECTIONS,
TOXLINE, DERWENT BIOTECHNOLOGY, EMBASE, PASCAL, APS, REGISTRY, CA
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Effect of Carrier Priming on Immunogenicity of Saccharide-Protein Conjugate Vaccines

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Previous studies with saccharide-protein conjugates have demonstrated that antibody responses to the saccharide can be improved by the preexistence of carrier immunity. Here we report that prior exposure to the carrier protein can either enhance or suppress antibody response to polysaccharides administered in saccharide-protein conjugates. A dose-dependent role for carrier priming in the antisaccharide antibody response to three saccharide-protein conjugate vaccines, i.e., a *Streptococcus pneumoniae* type 4 polysaccharide-tetanus toxoid (TT) conjugate (PS4TT), a *Neisseria meningitidis* group C polysaccharide-TT conjugate (MenCTT), and a *N. meningitidis* group C oligosaccharide-diphtheria mutant toxin conjugate (MenCCRM), was investigated. The results showed that an increase in the antipolysaccharide antibody response could be obtained for both PS4TT and MenCTT but not for MenCCRM with low-dose carrier priming (0.025 to 0.25 µg). However, suppression of the antipolysaccharide antibody response was observed with the PS4TT and MenCTT vaccines with high-dose (25-µg) carrier priming. There was no suppression effect with MenCCRM. The increase in the antipolysaccharide antibody response was shown to be restricted to the immunoglobulin G1 (IgG1) subclass, whereas suppression with high-dose carrier priming affected all antipolysaccharide subclass antibodies induced by PS4TT (IgG1, IgG2b, and IgG3) and only two of the four subclass antibodies induced by MenCTT (IgG2a and IgG2b). The increase in the antipolysaccharide antibody response was also present at the antipolysaccharide IgM antibody level but was not observed at the anti-carrier IgG antibody level.

The enhancement of the immunogenicity of polysaccharides by coupling polysaccharides or oligosaccharides to proteins has been amply demonstrated (1, 2, 4, 7, 9, 14). In most cases, saccharides have been coupled to large immunogenic proteins such as tetanus toxoid (TT) or diphtheria toxoid (DT). These proteins are chosen in most studies because both have been employed for human vaccination for many years without untoward side effects. Moreover, since most individuals are immunized with TT and DT, the response against a hapten coupled to carriers like TT and DT potentially can be improved by the preexistence of anticarrier immunity. However, the antibody response to a hapten coupled to the carrier protein can also be inhibited when the recipient has been previously immunized with the unmodified protein. This phenomenon has been termed carrier-induced epitope suppression (17) and was recently demonstrated to occur with a number of synthetic peptide-protein conjugates (10, 19, 20, 27, 28, 31). In humans, carrier-induced epitope suppression has been described for synthetic peptides coupled to TT (10, 14). Several investigators suggested that epitope suppression could also occur upon vaccination with saccharide-protein conjugate vaccines in human adults (7, 8, 16), but experimental evidence was not given. Carrier priming with one dose of carrier protein has been shown to have a positive effect on the polysaccharide response (8, 16).

The application of saccharide-protein conjugates is of primary importance in inducing protective immunity against infection with several encapsulated bacteria in infants and in elderly and immunodeficient patients. Therefore, we investigated in an animal model the role of carrier priming on the antibody response to a saccharide-protein conjugate. Since different saccharides can be coupled to TT, it is important to know whether the influence of carrier priming is similar with different saccharides when TT is used as a carrier. We found that preimmunization with low doses of TT enhanced the antibody response to *Streptococcus pneumoniae* type 4 polysaccharide (PS4) and meningococcal group C (MenC) polysaccharide upon vaccination with TT conjugates of these polysaccharides. High doses of carrier priming, however, inhibited a conjugate-induced antipolysaccharide antibody response. Carrier priming with DT did not affect an antibody response to a MenC oligosaccharide-mutant DT (CRM197) conjugate (MenCCRM197).

MATERIALS AND METHODS

Animals. One-week-old male and female and 8- to 12-week-old female NIH/RIVM mice (random outbred strain) were used. They were bred and kept at the National Institute of Public Health and Environmental Protection.

Antigens. In this study, three different saccharide-protein conjugates were used. A polysaccharide-TT conjugate of *S. pneumoniae* type 4 (PS4TT) with a polysaccharide protein ratio of 0.7:1 (wt/wt) was prepared by using the carbodiimide coupling procedure as described earlier (21). In brief, PS4 was activated with cyanogen bromide and subsequently coupled to a spacer 6-aminohexanoic acid. The purified

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product of this reaction was coupled to TT by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Polysaccharide-protein conjugates were purified by gel filtration on a Sepharose CL-4B column. The second polysaccharide-protein conjugate used was a conjugate of MenC and TT (MenCTT) with a polysaccharide/protein ratio of 1:1 (wt/wt). The conjugate was prepared by using methods similar to those described above for PS4TT (4).

In addition, an experimental oligosaccharide-mutant DT conjugate (CRM197) vaccine of *Neisseria meningitidis* group C (MenCCRM) was used. Oligosaccharides of MenC polysaccharide, obtained by mild periodate oxidation, were coupled to CRM197 with a saccharide-to-protein ratio of 1:2.5 by using reductive amination (2).

TT and DT were purchased from the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

Immunization scheme. Groups of 8 to 10 mice were immunized subcutaneously (s.c.) with 0.1-ml solutions of variable doses (ranging from 0.025 to 25 μ g) of TT or DT in 0.9% NaCl. A s.c. second immunization with the saccharide-protein conjugate containing 0.5 μ g of saccharide was given 42 days after carrier priming. Neonatal mice were primed with variable doses of TT at 1 week of age and were immunized with 0.5 μ g of conjugate (containing 0.5 μ g of saccharide) at days 28 and 56 after carrier priming. Blood samples were obtained from the tail veins at various times after immunization with the conjugate. Sera were stored at -20°C until use.

Antibody titers. Antibody titers against MenC polysaccharide, PS4, TT, and DT were determined by enzyme-linked immunosorbent assay as described below.

Wells of highly activated immunoassay microplates (Flow, Irvine, United Kingdom) were coated with rabbit anti-PS4 antibodies (State Serum Institute, Copenhagen, Denmark) in 0.05 M carbonate buffer (pH 9.6). After incubation for 3 h at 37°C , plates were washed and incubated overnight at 4°C with 1 μ g of PS4 (American Type Culture Collection, Rockville, Md.) per ml in 0.9% NaCl. For the detection of anti-MenC polysaccharide antibodies, microplates were coated with sheep antibodies to MenC polysaccharide (10 μ g/ml) (3) in 0.01 M phosphate-buffered saline (PBS; pH 7.2) overnight at room temperature, washed, and incubated with MenC polysaccharide (0.1 μ g/ml) (National Institute of Public Health and Environmental Protection) in PBS-Tween 80 (0.05%)-bovine serum albumin (0.5%) for 2 h at 37°C . For the determination of anticarrier antibody responses, microplates were coated with TT or DT (1 μ g/ml) in carbonate buffer (0.05 M, pH 9.6) overnight at room temperature. Subsequently, all coated plates were washed and then incubated for 2 h at 37°C with threefold serial dilutions of serum samples in PBS containing 0.05% (vol/vol) Tween 20 and 1% (wt/vol) bovine serum albumin. Plates were washed again and incubated for 2 h at 37°C with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Ig) antibodies (Southern Biotechnology Associates, Birmingham, Ala.), which detect IgG, IgM, IgG1, IgG2a, IgG2b, and IgG3. Plates were washed and incubated with the phosphatase substrate *p*-nitrophenylphosphate (Sigma, St. Louis, Mo.) at a concentration of 1 mg/ml in 10% diethanolamine buffer (pH 9.8). After 30 to 60 min of incubation at room temperature, the reaction was stopped by the addition of 50 μ l of 2.4 N NaOH and the A_{405} was read by using a Titertek Multiscan ELISA reader (Flow). All antibody titers were expressed as percentages of a hyperimmune serum either for PS4 or MenC polysaccharide or for TT or DT.

To exclude interference by antipneumococcal cell wall polysaccharide antibodies in the determination of IgM anti-PS4 antibodies, all sera were adsorbed with soluble cell wall polysaccharide (CPs; State Serum Institute). To that end, soluble cell wall polysaccharide at a concentration of 250 μ g/ml was added to the dilution buffer during serum incubation as described in detail elsewhere (22).

Statistical analysis. Results are expressed as logarithmic mean titers of n independent observations \pm standard deviation. Geometric mean (antilog) titers are expressed in parentheses. Significance was tested by a one-way or two-way analysis of variance. Dunnett's correction (12) was used to compare the different treatment groups with the control group.

RESULTS

Effect of preimmunization with carrier on antibody responses to polysaccharide-protein conjugates in adult mice. In order to investigate the dose dependency as well as the specificity of carrier priming, animals were immunized with various amounts (0.025 to 25 μ g) of carrier protein (TT or DT). Six weeks later, animals were immunized with 0.5 μ g of a saccharide-protein conjugate. Anti-polysaccharide IgG antibodies as measured on day 21 after immunization with the conjugate are shown in Table 1. Mice which were preimmunized with a low dose of carrier protein (0.025 μ g of TT) before administration of the polysaccharide-protein conjugates (MenCTT or PS4TT) had antipolysaccharide antibodies higher than those of mice injected with saline or an irrelevant carrier protein (DT). This effect was statistically significant for mice immunized with MenCTT ($P < 0.01$) after carrier priming but not for mice immunized with PS4TT after carrier priming. When mice were primed with a high dose of carrier protein (25 μ g) before immunization with the polysaccharide-protein conjugates, the antipolysaccharide antibody response was suppressed, unlike the response in mice primed with a high dose of irrelevant protein or saline. In mice immunized with PS4TT, the lower anti-PS4 antibody response was statistically significant ($P < 0.01$). Mice immunized with MenCTT after being primed with 25 μ g of TT showed a tendency to a lower antipolysaccharide antibody response; however, differences from their respective controls were not significant. In contrast to results with the polysaccharide-TT conjugates, no effect of carrier priming on the antipolysaccharide IgG antibody response induced by the oligosaccharide-protein conjugate vaccine MenCCRM could be observed after priming at any dose level of DT. Anti-TT IgG antibody responses in mice primed with 25 μ g of TT and reimmunized with PS4TT did not differ from those of mice primed with 2.5 μ g of TT (Fig. 1). Either low- or high-dose carrier priming enhanced the anti-PS4 IgM antibody response ($P < 0.01$) but did not affect anti-MenC polysaccharide IgM antibody responses (Table 2). The inhibiting effect of high-dose (25- μ g) carrier priming was thus limited to the antipolysaccharide IgG antibody response.

Effect of preimmunization with carrier on antibody responses to a polysaccharide-protein conjugate in neonatal mice. Of interest is whether the effects in neonatal mice of carrier priming on the polysaccharide antibody response, when the polysaccharide is administered as part of a polysaccharide-protein conjugate, are similar to the effects observed in adult mice. Neonatal mice were immunized with variable doses of TT at 1 week of age and then with 0.5 μ g of PS4TT at 5 weeks of age. As in adult mice, low-dose carrier priming (0.025 μ g of TT) of neonatal mice enhanced a

TABLE 1. Dose-dependent effects of carrier priming on antipolysaccharide IgG antibody level to saccharide-protein conjugates in adult mice

Primary immunization and amt (μ g) ^a	Secondary immunization ^b	Mean log ₁₀ titer \pm SD (GMT) ^c	No. of responding mice/total no. of mice tested
Saline	PS4TT	2.079 \pm 0.856 (120)	8/9
TT			
0.025	PS4TT	2.735 \pm 0.442 (544)	10/10
0.25	PS4TT	2.730 \pm 0.444 (537)	10/10
2.5	PS4TT	1.568 \pm 0.582 (37)	7/10
25	PS4TT	1.000 \pm 0.723 (10)	5/10 ^d
DT			
0.025	PS4TT	2.225 \pm 0.746 (168)	10/10
0.25	PS4TT	2.393 \pm 0.804 (247)	9/10
2.5	PS4TT	2.097 \pm 0.833 (125)	9/10
25	PS4TT	2.143 \pm 0.698 (139)	9/10
Saline	MenCTT	2.519 \pm 0.413 (416)	10/10
TT			
0.025	MenCTT	3.412 \pm 0.250 (2,583)	10/10 ^d
0.25	MenCTT	3.145 \pm 0.396 (1,395)	10/10 ^d
2.5	MenCTT	2.491 \pm 0.290 (310)	10/10
25	MenCTT	2.238 \pm 0.279 (173)	10/10
DT			
0.025	MenCTT	2.677 \pm 0.276 (475)	10/10
0.25	MenCTT	2.599 \pm 0.294 (397)	10/10
2.5	MenCTT	2.816 \pm 0.427 (655)	10/10
25	MenCTT	2.413 \pm 0.436 (259)	10/10
Saline	MenCCRM	2.117 \pm 0.648 (131)	10/10
TT			
0.025	MenCCRM	2.164 \pm 0.364 (146)	10/10
0.25	MenCCRM	1.851 \pm 0.326 (71)	10/10
2.5	MenCCRM	1.832 \pm 0.348 (68)	10/10
25	MenCCRM	1.881 \pm 0.393 (76)	10/10
DT			
0.025	MenCCRM	1.875 \pm 0.419 (75)	10/10
0.25	MenCCRM	2.428 \pm 0.396 (268)	10/10
2.5	MenCCRM	2.382 \pm 0.253 (241)	10/10
25	MenCCRM	1.996 \pm 0.499 (99)	10/10

^a Groups of 8 to 10 mice were immunized s.c.

^b Mice were immunized s.c. with 0.5 μ g of saccharide-protein conjugate on day 42 after immunization with the carrier.

^c Expressed as percentage of a reference serum at day 21 after immunization with the conjugate vaccine. GMT, geometric mean titer (antilog).

^d $P < 0.01$. P value was based on comparisons with controls not immunized with TT or DT prior to vaccination with the conjugate vaccine.

conjugate-induced anti-PS4 antibody response by both the IgM and IgG isotypes (Fig. 2). High-dose carrier priming resulted in suppression of the conjugate-induced anti-PS4 IgG antibody response. The phenomena of enhancement and inhibition of anti-PS4 antibody formation after carrier priming therefore were operative in both adult and neonatal mice. Neonatal mice were immunized a second time with PS4TT at 9 weeks of age, and this immunization resulted in an 8.4-fold increase in anti-PS4 IgG antibodies in low-dose (0.025- μ g)-carrier-primed mice. In nonprimed animals, the anti-PS4 IgG antibody response increased only 3.6-fold upon a second immunization with PS4TT (Fig. 2). In high-dose-carrier-primed mice, no increase in either PS4 IgM or PS4 IgG antibodies could be observed upon secondary immunization with the conjugate (Fig. 2). The consequences of carrier priming thus remained clear even after repeated immunization with the conjugate vaccines. Statistical analysis of data

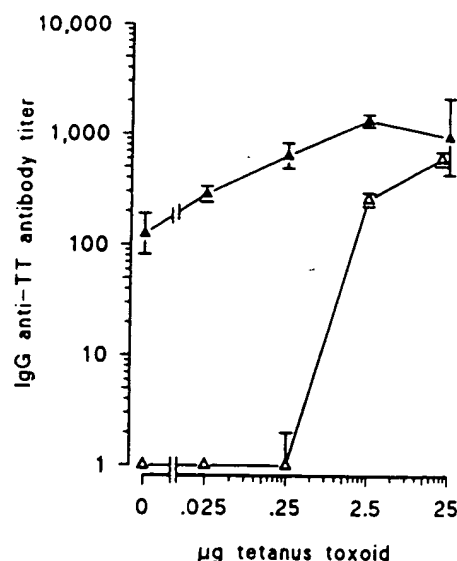


FIG. 1. Dose-dependent influence of TT priming on the anti-TT IgG antibody response to a polysaccharide-protein conjugate in adult mice. Groups of 10 mice were immunized with various amounts of TT (0 to 25 μ g). Anti-TT IgG antibodies were analyzed at day 28 after immunization (Δ). All mice were reimmunized with PS4TT, containing 0.5 μ g of saccharide, at day 28 after immunization with the carrier. Anti-TT IgG antibodies were also analyzed at day 21 after injection with the conjugate (\bullet). Anti-TT IgG antibody titers are shown as geometric means \pm 1 standard deviation.

from both neonatal and adult mice showed a significant increase in the anti-PS4 IgG antibody response ($P < 0.01$) of low-dose-carrier-primed mice reimmunized with the conjugate and a significant decrease ($P < 0.05$) in the anti-PS4 IgG response of high-dose-carrier-primed mice reimmunized with the conjugate.

Influence of TT priming on IgG subclass distribution of antipolysaccharide antibodies. Preimmunization with TT may also influence the IgG subclass distribution of the antipolysaccharide antibody response. Results in Table 3 show that upon low-dose carrier priming, the increased levels of anti-MenC polysaccharide IgG antibodies were mainly due to an increase in the IgG1 subclass ($P < 0.01$). The IgG2b subclass increased to a lesser extent ($P < 0.05$), whereas the IgG3 antibody level was significantly decreased ($P < 0.05$). No effect on the IgG2a antibody level was observed with low-dose carrier priming. The increase in IgG1 antibodies was also observed with PS4TT but not with the oligosaccharide-protein conjugate MenCCRM. High-dose carrier priming showed suppression of all IgG subclasses (IgG1, IgG2b, and IgG3) ($P < 0.05$) with the PS4TT vaccine, whereas suppression of the antipolysaccharide immune response to MenCTT (which induces IgG1, IgG2a, IgG2b, and IgG3 antibodies) was evident only for the IgG2a ($P < 0.01$) and IgG2b antibodies. No effect of high-dose carrier priming was observed with the oligosaccharide-protein conjugate vaccine which induced predominantly antipolysaccharide antibodies of the IgG1 subclass.

In neonatal mice, the effects of low- and high-dose carrier priming on the antipolysaccharide IgG subclass distribution upon immunization with the PS4TT vaccine were similar to those found in adult mice (data not shown).

TABLE 2. Effect of carrier priming on antipolysaccharide IgM antibody level to saccharide-protein conjugates in adult mice

Primary immunization and amt (μ g) ^a	Secondary immunization ^b	Mean log ₁₀ titer \pm SD (GMT) ^c	No. of responding mice/total no. of mice tested
Saline	PS4TT	0.954 \pm 0.279 (9)	8/9
TT			
0.025	PS4TT	1.740 \pm 0.265 (55)	6/6 ^d
0.25	PS4TT	1.663 \pm 0.188 (46)	10/10 ^d
2.5	PS4TT	1.491 \pm 0.252 (31)	10/10 ^d
25	PS4TT	1.568 \pm 0.344 (37)	8/8 ^d
DT			
0.025	PS4TT	1.000 \pm 0.338 (10)	7/8
0.25	PS4TT	1.322 \pm 0.217 (21)	10/10
2.5	PS4TT	1.113 \pm 0.399 (13)	7/10
25	PS4TT	0.954 \pm 0.431 (9)	8/10
Saline	MenCTT	2.364 \pm 0.207 (231)	9/9
TT			
0.025	MenCTT	2.472 \pm 0.236 (297)	10/10
0.25	MenCTT	2.399 \pm 0.143 (251)	10/10
2.5	MenCTT	1.857 \pm 0.207 (72)	8/8
25	MenCTT	1.991 \pm 0.201 (98)	10/10
DT			
0.025	MenCTT	2.272 \pm 0.127 (187)	10/10
0.25	MenCTT	2.410 \pm 0.292 (257)	10/10
2.5	MenCTT	2.332 \pm 0.301 (215)	10/10
25	MenCTT	2.232 \pm 0.326 (171)	10/10
Saline	MenCCRM	1.397 \pm 0.250 (25)	9/9
TT			
0.025	MenCCRM	1.176 \pm 0.312 (15)	8/10
0.25	MenCCRM	1.114 \pm 0.265 (13)	8/10
2.5	MenCCRM	1.230 \pm 0.250 (17)	9/10
25	MenCCRM	1.041 \pm 0.190 (11)	8/10
DT			
0.025	MenCCRM	1.362 \pm 0.538 (23)	8/10
0.25	MenCCRM	1.875 \pm 0.378 (75)	10/10
2.5	MenCCRM	1.633 \pm 0.449 (43)	10/10
25	MenCCRM	1.255 \pm 0.350 (18)	9/10

^a Groups of 8 to 10 mice were immunized s.c.^b Mice were immunized s.c. with saccharide-protein conjugate, containing 0.5 μ g of saccharide, at day 42 after immunization with the carrier.^c Expressed as percentage of a reference serum at day 21 after immunization with the conjugate vaccine. GMT, geometric mean titer (antilog).^d $P < 0.01$. P values were based on comparisons with controls not immunized with TT or DT prior to vaccination with the conjugate vaccine.

DISCUSSION

This study was performed to investigate whether carrier priming affects the antibody response to polysaccharides when DT or TT is used as a carrier protein in saccharide-protein conjugate vaccines. It is known from earlier studies with hapten-carrier and synthetic peptide-carrier conjugate vaccines that the antibody response to the hapten conjugated to a protein can be either suppressed or enhanced by prior immunization with the carrier protein alone. Previous observations in mice have shown that preimmunization with the carrier protein and a *Haemophilus influenzae* type b polysaccharide-bovine serum albumin conjugate enhances the antipolysaccharide antibody response (24). Similar observations have been made by other investigators who used the same saccharide and the pneumococcal serotype 6A capsular polysaccharide coupled to other carrier proteins (1, 3, 6, 25, 26). The phenomenon of epitope suppression has been described for haptens, small peptides, and polymeric syn-

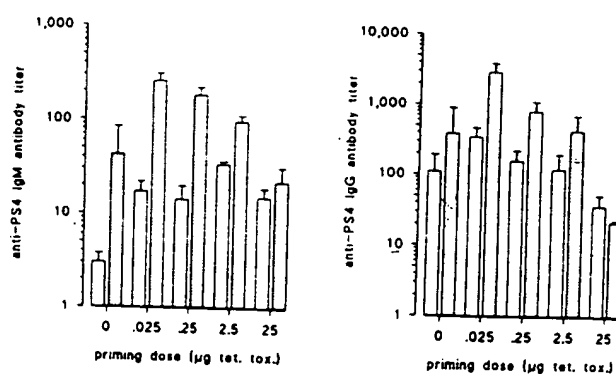


FIG. 2. Dose-dependent influence of carrier priming on anti-PS4 IgG and IgM antibody levels after two vaccinations with PS4TT in neonatal mice. Groups of 8 to 10 1-week-old mice were immunized with various amounts of TT (0 to 25 μ g per animal). Four weeks after immunization with the carrier, all mice were vaccinated with 0.5 μ g of PS4TT. Anti-PS4 IgM (left) was analyzed in individual serum samples at day 8 following immunization, and anti-PS4 IgG antibodies (right) were analyzed at day 28 after immunization (open bars). A second vaccination with the conjugate was given 4 weeks after primary immunization with the conjugate. Anti-PS4 IgM and anti-PS4 IgG antibodies were analyzed in individual serum samples on days 8 and 21, respectively, after the second vaccination with the conjugate (shaded bars).

thetic peptides coupled to the carrier protein but not for polysaccharides or oligosaccharides until now. Our data indicate that apart from enhancement of the antisaccharide antibody response, suppression can also occur by carrier priming when high-molecular-weight capsular polysaccharides are coupled to proteins. In a number of other systems, it has been demonstrated that concomitant immunization with carrier protein and conjugate vaccine does lead to a higher antipolysaccharide response (6, 25). Enhancement of the antipolysaccharide antibody response was observed in our studies by low-dose carrier priming for PS4TT and MenCTT. It was observed that low-dose carrier priming especially increased IgG1 and IgG2b antipolysaccharide antibodies. Since both IgG1 and IgG2b (IgG1 \gg IgG2b) are subclasses restricted to a typical T-cell-dependent antibody response in mice (29), it might be suggested that an increase in carrier-specific T-cell help resulted in a more T-cell-dependent antibody response. Antibodies of the IgG3 subclass are predominantly induced in an immune response to T-cell-independent type 2 antigens (such as polysaccharides) and were found in minor quantities upon vaccination with PS4TT and MenCTT. Antibodies of this subclass were observed to be decreased after low-dose carrier priming and subsequent vaccination with MenCTT. This might indicate that the antipolysaccharide antibody response indeed switched to a more T-cell-dependent character. Remarkable was that the doses of TT used for priming that caused enhancement of the antipolysaccharide response induced by TT-conjugated polysaccharide did not induce detectable anti-TT antibody levels after primary immunization (Fig. 1). Increasing the priming dose of carrier to 2.5 μ g per animal, which results in detectable anti-TT antibodies, abrogated the positive effect of carrier priming on the antipolysaccharide antibody response. In contrast to earlier observations, we were able to show that using a high dose of TT (25 μ g per animal) administered as PS4TT or MenCTT for a priming dose suppressed the antipolysaccharide IgG antibody response. In agreement with data in the literature on hapten-

TABLE 3. Effect of carrier priming on IgG subclass distribution of antipolysaccharide antibodies

Primary immunization and amt (μ g) ^a	Secondary immunization ^b	Mean log ₁₀ titer \pm SD (GMT) for ^c :			
		IgG1	IgG2a	IgG2b	IgG3
Saline	PS4TT	2.076 \pm 0.857 (119)		0.778 \pm 1.109 (6)	1.255 \pm 0.728 (18)
TT					
0.025	PS4TT	2.759 \pm 0.493 (574)		1.204 \pm 0.469 (16)	1.114 \pm 0.474 (13)
0.25	PS4TT	2.740 \pm 0.480 (550)		1.415 \pm 0.469 (26)	1.146 \pm 0.393 (14)
2.5	PS4TT	1.462 \pm 0.588 (29)		0 (1)	0.301 \pm 0.401 (2)
25	PS4TT	0.778 \pm 0.709 (6)		0 (1)	0.477 \pm 0.487 (3)
DT					
0.025	PS4TT	2.149 \pm 0.833 (141)		1.079 \pm 0.865 (12)	1.041 \pm 0.478 (11)
0.25	PS4TT	2.283 \pm 1.276 (192)		1.255 \pm 0.582 (18)	1.146 \pm 0.506 (14)
2.5	PS4TT	1.919 \pm 1.273 (83)		1.041 \pm 0.677 (11)	1.000 \pm 0.906 (10)
25	PS4TT	2.021 \pm 0.741 (105)		0.778 \pm 0.792 (6)	1.362 \pm 0.602 (23)
Saline	MenCTT	2.584 \pm 0.459 (384)	1.301 \pm 0.468 (20)	1.230 \pm 0.418 (17)	2.152 \pm 0.334 (142)
TT					
0.025	MenCTT	3.420 \pm 0.340 (2,632)	1.114 \pm 0.441 (13)	1.785 \pm 0.382 (61)	1.380 \pm 0.333 (24)
0.25	MenCTT	3.157 \pm 0.371 (1,436)	0.778 \pm 0.558 (6)	1.230 \pm 0.283 (17)	1.342 \pm 0.290 (22)
2.5	MenCTT	2.393 \pm 0.272 (247)	1.000 \pm 0.792 (10)	1.301 \pm 0.559 (20)	1.813 \pm 0.380 (65)
25	MenCTT	2.267 \pm 0.322 (185)	0 (1)	0.301 \pm 1.123 (2)	1.612 \pm 0.464 (41)
DT					
0.025	MenCTT	2.694 \pm 0.299 (494)	1.380 \pm 0.401 (24)	1.322 \pm 0.742 (21)	2.053 \pm 0.238 (113)
0.25	MenCTT	2.619 \pm 0.344 (416)	1.079 \pm 0.800 (12)	1.079 \pm 0.497 (12)	1.839 \pm 0.238 (69)
2.5	MenCTT	2.829 \pm 0.525 (674)	1.643 \pm 0.462 (44)	1.256 \pm 0.621 (18)	2.100 \pm 0.210 (126)
25	MenCTT	2.459 \pm 0.429 (288)	0.778 \pm 0.722 (2)	1.000 \pm 0.436 (10)	1.778 \pm 0.410 (60)
Saline	MenCCRM	2.152 \pm 0.613 (142)			
TT					
0.025	MenCCRM	2.217 \pm 0.367 (165)			
0.25	MenCCRM	1.886 \pm 0.344 (77)			
2.5	MenCCRM	1.892 \pm 0.328 (78)			
25	MenCCRM	1.899 \pm 0.386 (79)			
DT					
0.025	MenCCRM	1.908 \pm 0.410 (81)			
0.25	MenCCRM	2.507 \pm 0.420 (321)			
2.5	MenCCRM	2.407 \pm 0.246 (255)			
25	MenCCRM	2.068 \pm 0.545 (117)			

^a Individual serum samples from 10 mice per experimental group were analyzed for antipolysaccharide IgG antibodies 3 weeks after immunization with the saccharide-protein conjugates. Mice were primed with various amounts of TT or DT (0 to 25 μ g per animal).

^b Six weeks after immunization with the carrier, mice were immunized with saccharide-protein conjugates, containing 0.5 μ g of saccharide.

^c Given as percentage of a hyperimmune serum. GMT, geometric mean titer (antilog). Anti-PS4 IgG antibodies of the IgG2a subclass were not increased compared with antibodies in nonimmune sera when mice were immunized with PS4TT after carrier priming. Only anti-MenC polysaccharide antibodies of the IgG1 subclass were enhanced compared with antibodies in nonimmune sera when mice were immunized with MenCCRM after carrier priming.

carrier and peptide-carrier conjugate studies, we could not observe an influence of high-dose carrier priming on the antisaccharide IgM antibody level. It has been suggested (8) that the use of high-dose carrier priming led to antihapten antibody responses with a T-cell-independent character. Since most polysaccharides are T-cell-independent type 2 antigens, the T-cell-independent character of the antipolysaccharide antibody response under epitope-suppressed conditions should be very clear in this study. However, analysis of the antipolysaccharide IgG subclass distribution showed that all three subclasses (IgG1, IgG2b, and IgG3) which could be induced by PS4TT were suppressed upon high-dose carrier priming. With MenCTT, only IgG2a and IgG2b of the four contributing subclasses (IgG1, IgG2a, IgG2b, and IgG3) were significantly suppressed. The observation that only IgG2a and IgG2b were suppressed by high-dose carrier priming was reported earlier by Herzenberg et al. (17, 18). Our data do not support the hypothesis that the hapten behaves as a T-cell-independent antigen, since the IgG3 subclass, the antibody subclass preferentially induced by T-cell-independent antigens, would not be influ-

enced by high-dose carrier priming. No indication for either low-dose-carrier-induced enhancement or high-dose-carrier-induced inhibition of the antipolysaccharide antibody response could be observed when a DT-MenCCRM combination was used. In contrast to results in the study by Vella and Ellis (30), no DT priming was necessary for the generation of an antipolysaccharide antibody response with an oligosaccharide-mutant DT conjugate vaccine. CRM197 differs from DT at a single amino acid position (15). It has, however, been demonstrated that there is a high degree of antibody cross-reaction (23) and T-cell cross-reactivity (5) between CRM197 and DT. The lack of epitope suppression in the DT-MenCCRM combination is therefore unlikely to be due to minor antigenic differences between the protein used for priming and the carrier protein of the conjugate. The absence of epitope suppression might be due to the lower immunogenicity of DT compared with the immunogenicity of TT in NIH/RIVM mice; after primary immunization of mice with DT, we could hardly detect antibodies against DT even with the highest dose (25 μ g) of DT used (geometric mean titer, <1). Another explanation might be the difference in confor-

mation and/or saccharide size of MenCCRM compared with those of MenCTT and PS4TT, which are prepared according to a similar coupling procedure and which contain the whole capsular polysaccharide. To our knowledge, there are two other studies in which the influence of high-dose DT priming on the antibody response to a peptide-DT or a saccharide-protein conjugate was reported (1, 14). In one study, DT also failed to induce epitope suppression, whereas when TT was used as a carrier protein to the same peptide, suppression was induced (14). In the study by Anderson (1), an improved antipolysaccharide antibody response occurred upon vaccination with the *H. influenzae* type b oligosaccharide-mutant DT conjugate in rabbits primed with 100 µg of carrier protein on aluminum phosphate adjuvant compared with nonprimed animals.

In this study, we have used two TT-polysaccharide conjugates and one CRM197-oligosaccharide conjugate, which is apart from carrier protein and is also constructed differently from the two TT conjugates. Therefore, variables such as the nature of the polysaccharide, the protein/polysaccharide ratio, and unidentified factors related to the particular construction and shape may have contributed to the inhibitory effects observed. No general rule on the effects of carrier priming can therefore be deduced from these experiments. The data do indicate, however, that high-dose carrier priming may cause significant suppressive effects on the antipolysaccharide IgG antibody response induced by conjugated polysaccharide.

The mechanism responsible for suppression of the anti-hapten antibody response to hapten-carrier conjugates upon high-dose carrier priming is still unclear. Involvement of both T suppressor cells (13, 17-19, 27, 28) and carrier-specific B memory cells (13, 19, 27, 28) has been reported. Herzenberg et al. (17, 18) postulated activation of T suppressor cells. Schutze et al. (27, 28) showed the involvement of B memory cells in the phenomenon of epitope suppression. They argue that because of prior immunization with the carrier protein, a predominance of carrier-specific B-cell clones exists that competitively decreases the interaction between hapten-specific B cells and the hapten at the time of conjugate immunization. Another possible explanation might be that circulating antibodies against the carrier protein are responsible for scavenging of the antigen or the formation of immune complexes which inhibit the immune response.

Recent studies with *H. influenzae* type b polysaccharide-oligosaccharide-protein conjugates show that these conjugate vaccines are highly efficacious in preventing *H. influenzae* type b disease in infants and young children (32). These conjugate vaccines as well as new conjugate vaccines under development will therefore be used on a wide scale in the near future in order to protect children against infections by several encapsulated bacteria. In most cases, DT or TT will be used as the carrier protein in the conjugate vaccine. Because of the growing emphasis on TT immunizations in different health programs, it is important to be aware of a potential negative effect of the vaccination status of humans in their response to those conjugate vaccines. However, extrapolation of our observed data to the human situation should be made cautiously for several reasons. First, the induction of epitope suppression in mice required relatively high doses of carrier protein relative to body weight (25 µg per animal), since only 10 to 15 µg is used for immunizations in humans. However, we do not know whether sensitization by repeated injections with lower doses of TT can also induce epitope suppression. Second, in our study, mice were

immunized only with TT, whereas in humans, TT is administered together with diphtheria, pertussis, and polio vaccines in a combination vaccine. Two studies in mice have shown that the use of *Bordetella pertussis* vaccine and its adjuvant components can modulate immune suppression (17, 31). Thus, it is possible that the pertussis component in the combination vaccine diminishes the potential for TT to cause epitope suppression in humans. Clearly, more studies are needed to address the effect of carrier priming on the outcome of saccharide-protein conjugate vaccinations as well as to develop different ways of circumventing epitope suppression.

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REFERENCES

- Anderson, P. 1983. Antibody responses to *Haemophilus influenzae* type b and diphtheria toxin induced by conjugate of oligosaccharides of the type b capsule with the non-toxic protein CRM197. *Infect. Immun.* 39:233-238.
- Anderson, P. W., M. E. Pichichero, R. A. Insel, R. Betts, R. Eby, and D. H. Smith. 1986. Vaccines consisting of periodate cleaved oligosaccharides from the capsule of *H. influenzae* type b coupled to a protein carrier: structural and temporal requirements for priming in the human infant. *J. Immunol.* 137:1181-1186.
- Barington, T., K. Kristensen, J. Henrichsen, and C. Heilmann. 1991. Influence of prevaccination immunity on the human B lymphocyte response to a *Haemophilus influenzae* type b conjugate vaccine. *Infect. Immun.* 59:1057-1064.
- Beuvery, E. C., F. van Rossum, and J. Nagel. 1982. Comparison of the induction of immunoglobulin M and G antibodies in mice with purified pneumococcal type 3 and meningococcal group C polysaccharides and their protein conjugates. *Infect. Immun.* 37:15-22.
- Bixler, G. S., and S. Pillai. 1989. The cellular basis of the immune response by conjugate vaccines. *Contrib. Microbiol. Immunol.* 10:18-47.
- Chu, C., R. Schneerson, J. B. Robbins, and S. C. Rastogi. 1983. Further studies on the immunogenicity of *Haemophilus influenzae* type b and pneumococcal type 6A polysaccharide-protein conjugate. *Infect. Immun.* 40:245-256.
- Cryz, S. J., J. C. Sadoff, E. Fürer, and R. Germanier. 1986. *Pseudomonas aeruginosa* polysaccharide-tetanus toxoid conjugate vaccine: safety and immunogenicity in humans. *J. Infect. Dis.* 154:682-688.
- Di John, D., J. R. Torres, J. Murillo, E. A. Herrington, S. S. Wasserman, M. J. Lasonsky, D. Stürker, and M. M. Levine. 1989. Effect of priming with carrier on response to conjugate vaccine. *Lancet* ii:1415-1418.
- Einhorn, M. S., G. A. Weinberg, E. L. Anderson, G. A. Weinberg, P. D. Granoff, and D. M. Granoff. 1986. Immunogenicity in infants of *H. influenzae* type b polysaccharide in a conjugate vaccine with *Neisseria meningitidis* outer-membrane protein. *Lancet* ii:299-302.
- Etlinger, H. M., A. M. Felix, D. Lillessen, E. D. Haemer, M. Just, J. R. L. Pink, F. Sinigaglia, D. Stürchler, B. Tacaks, A. Trzeciak, and H. Matile. 1988. Assessment in humans of a synthetic peptide-based vaccine against the sporozoite stage of the human malaria parasite, *Plasmodium falciparum*. *J. Immunol.* 140:626-633.
- Etlinger, H. M., D. Gillessen, H. W. Lahm, H. Matile, H. J. Schönfeld, and A. Trzeciak. 1990. Use of prior vaccinations for the development of new vaccines. *Science* 249:423-425.
- Fleiss, J. L. 1986. The design and analysis of clinical experi-

- ments. John Wiley & Sons. Chichester, England.
13. Galleli, A., and B. Charlot. 1990. Clonal anergy of memory B cells in epitope specific regulation. *J. Immunol.* 145:2397-2405.
14. Gaur, A., K. Arunan, O. Singh, and G. P. Talwar. 1990. Bypass by an alternate carrier of acquired unresponsiveness to hCG upon repeated immunization with tetanus-conjugated vaccine. *Int. Immunol.* 2:151-155.
15. Giannini, G., R. Rappuoli, and G. Ratti. 1984. The amino sequence of two non-toxic mutants of diphtheria toxin: CRM45 and CRM197. *Nucleic Acids Res.* 12:4063-4069.
16. Granoff, D. M., E. G. Boies, and R. S. Munson. 1984. Immunogenicity of *H. influenzae* type b polysaccharide diphtheria conjugate vaccines in adults. *J. Pediatr.* 105:22-27.
17. Herzenberg, L. A., and T. Tokuhisa. 1982. Epitope-specific regulation. I. Carrier-specific induction of suppression for IgG anti-hapten antibody responses. *J. Exp. Med.* 155:1730-1740.
18. Herzenberg, L. A., T. Tokuhisa, and K. Hayakawa. 1983. Epitope-specific regulation. *Annu. Rev. Immunol.* 1:609-632.
19. LeClerc, C., M. P. Schutze, E. Deriaud, and G. Przewlocki. 1990. The in vivo elimination of CD4⁺ T cells prevents the induction but not the expression of carrier-induced epitopic suppression. *J. Immunol.* 145:1343-1349.
20. Lise, L. D., D. Mazier, M. Jolivet, F. Audibert, L. Chedid, and D. Schlesinger. 1987. Enhanced epitopic response to a synthetic human malarial peptide by preimmunization with tetanus toxoid carrier. *Infect. Immun.* 55:2658-2661.
21. Peeters, C. C. A. M., A. J. Tenbergen-Meekes, D. E. Evenberg, J. T. Poolman, B. J. M. Zegers, and G. T. Rijkers. 1991. A comparative study of the immunogenicity of pneumococcal type 4 polysaccharide- and oligosaccharide-tetanus toxoid conjugates in adult mice. *J. Immunol.* 146:4308-4314.
22. Peeters, C. C. A. M., A. J. Tenbergen-Meekes, J. T. Poolman, B. J. M. Zegers, and G. T. Rijkers. Submitted for publication.
23. Porro, M., M. Saletti, L. Neucioni, L. Tagliaferri, and L. Marsili. 1980. I. Immunogenic correlation between cross-reacting material (CRM197) produced by a mutant *C. diphtheria* and diphtheria toxoid. *J. Infect. Dis.* 142:716-724.
24. Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins. 1980. Preparation, characterization and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. *J. Exp. Med.* 152:360-376.
25. Schneerson, R., J. B. Robbins, C. Chu, A. Sutton, W. Vann, J. C. Vickers, W. T. London, B. Curfman, M. C. Hardegree, J. Shiloach, and S. C. Rastogi. 1984. Serum antibody responses of juvenile and infant rhesus monkeys injected with *Haemophilus influenzae* type b and pneumococcus type 6A capsular polysaccharide-protein conjugates. *Infect. Immun.* 45:582-591.
26. Schneerson, R., J. B. Robbins, J. C. Parke, C. Bell, J. J. Schlesselman, A. Sutton, Z. Wang, G. Schiffman, A. Karpas, and J. Shiloach. 1986. Quantitative and qualitative analysis of serum antibodies elicited in adults by *H. influenzae* type b and pneumococcus 6A capsular polysaccharide-TT conjugates. *Infect. Immun.* 52:519-528.
27. Schutze, M. P., E. Deriaud, G. Przewlocki, and C. LeClerc. 1989. Carrier-induced epitopic suppression is initiated through clonal dominance. *J. Immunol.* 142:2635-2640.
28. Schutze, M. P., C. LeClerc, M. Jolivet, F. Audibert, and L. Chedid. 1985. Carrier-induced epitopic suppression, a major issue for synthetic vaccines. *J. Immunol.* 135:2319-2322.
29. Slack, J., G. P. Der Balian, M. Nahm, and J. M. Davie. 1980. Subclass restriction of murine antibodies. II. The IgG plaque forming cell response to thymus independent type 1 and type 2 antigens in normal mice and mice expressing an X-linked immunodeficiency. *J. Exp. Med.* 151:853-862.
30. Vella, P. P., and R. W. Ellis. 1991. Immunogenicity of *Haemophilus influenzae* type b conjugate vaccines in infant rhesus monkeys. *Pediatr. Res.* 29:10-13.
31. Vogel, F. R., C. LeClerc, M. P. Schutze, M. Jolivet, F. Audibert, T. W. Klein, and L. Chedid. 1987. Modulation of carrier-induced epitopic suppression by *Bordetella pertussis* components and muramyl peptide. *Cell. Immunol.* 107:40-51.
32. Ward, J., and S. Cochi. 1988. *Haemophilus influenzae*. p. 300-332. In S. A. Plotkin and E. A. Mortimer (ed.), *Vaccines*. The W. B. Saunders Co., Philadelphia.

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Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens

John D. Clements*, Nancy M. Hartzog and Frank L. Lyon†

The ability of *Escherichia coli* heat-labile enterotoxin (LT) to influence the induction and maintenance of tolerance was examined in animals primed orally with a soluble protein antigen, ovalbumin (OVA), or in animals primed orally with two unrelated protein antigens administered simultaneously, OVA and bovine serum albumin (BSA). LT is immunologically and structurally related to the cholera enterotoxin (CT), which has been shown to be capable of abrogating oral tolerance to protein antigens when delivered simultaneously with the antigens. In this study, simultaneous administration of LT with OVA was shown to prevent the induction of tolerance to OVA and to increase the serum anti-OVA IgG response 30- to 90-fold over OVA-primed and PBS-primed animals, respectively. This effect was determined to be a function of the enzymatically active A subunit of the toxin since the B (binding) subunit alone was unable to influence tolerance induction. Animals fed LT with OVA after the initial OVA prime developed a significantly lower serum IgG and mucosal IgA anti-OVA response than those fed LT with OVA in the initial immunization, indicating that prior exposure to the antigen reduces the effectiveness of LT to influence tolerance and its ability to act as an adjuvant. LT was not able to abrogate tolerance once it had been established. Serum IgG and mucosal IgA responses in animals receiving LT on only a single occasion, that being upon first exposure to antigen, were equivalent to responses after three OVA/LT primes, indicating that commitment to responsiveness occurs early and upon first exposure to antigen. An additional finding of this study was that LT can serve as an adjuvant for orally administered antigens and that it elicits the production of both serum IgG and mucosal IgA against antigens with which it is delivered.

Keywords: Adjuvant; tolerance; *Escherichia coli* enterotoxin; cholera toxin

Introduction

Oral immunization can lead to loss of systemic reactivity in response to subsequent parenteral injection of the specific antigen¹. This phenomenon, known as tolerance, has been shown to occur in numerous animal models and with a variety of antigens. Tolerance can be either complete or partial, and is influenced by antigen dose and characteristics, route of administration, physiological state of the organism and genetic characteristics of the organism. It has also been shown that tolerance can be terminated or prevented by various manipulations, depending upon the cellular basis of the state of tolerance.

It has recently been demonstrated that administration of cholera toxin (CT) can abrogate oral tolerance to an unrelated antigen². CT, an 84 000 dalton polymeric protein produced by *Vibrio cholerae*, consists of

two subunits, designated A and B. The B subunit binds the toxin to its cell surface receptor, the monosialosylganglioside GM₁, and facilitates the penetration of the toxic A subunit into the cell. The A subunit catalyses the ADP-ribosylation of the stimulatory GTP-binding protein (G_s) in the adenylate cyclase enzyme complex and this results in increasing intracellular levels of adenosine 3',5'-monophosphate (cAMP)^{3,4}. Some strains of *Escherichia coli* produce an immunologically and structurally related heat-labile enterotoxin (LT) that has the same subunit organization and arrangement as CT and that works by the same mechanism of action^{5,6}. Although there are many similarities between CT and LT, there are also immunological and structural differences between the two molecules^{5,6}. Moreover, the relative immunoregulatory potential of LT has not been thoroughly investigated. We have extensively studied LT and have recently developed a clone of *E. coli* that produces just the binding subunit of the toxin (LT-B)^{7,8}.

Although the mechanism for abrogation of tolerance by CT is unknown, it is presumed to result from an alteration of the regulatory environment in the gut

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lated epididymal fat cells from rats^{12,13}, elevate cAMP levels of intestinal tissues *in vivo*¹⁴, increase $\Delta^4,3$ -ketosteroids and induce morphological alterations in cultured mouse Y-1 adrenal tumour cells^{15,16}, and to increase accumulation of cAMP and induce morphological alterations in cultured Chinese hamster ovary cells¹⁷. Cultured fibroblasts respond with increased cAMP and increased collagen synthesis¹⁷, cell elongation and adhesion to substrate¹⁸, as well as by inhibition of nucleotide and amino acid transport and protein synthesis. These toxins have also been shown to stimulate basal adenylate cyclase activity in liver with a concomitant decrease in hepatic glycogen¹⁹, to stimulate adenylate cyclase activity of human embryonic intestinal epithelial cells in culture^{20,21}, and to increase membrane adenylate cyclase activity in mouse thymocytes²² and rat pituitary cells²³. Presumably because of the ubiquity of the G_{M1} ganglioside in cell membranes, CT and LT have been found to have a broad spectrum of activity and, in fact, elevate intracellular levels of cAMP in virtually every mammalian tissue tested²⁰.

It was recently discovered that simultaneous administration of LT with an orally administered antigen can also influence the development of tolerance to that antigen. This paper investigates the parameters of this effect and the relative contributions of the two subunits of the enterotoxin.

Materials and methods

Animals

Female Balb/c mice, four to six weeks of age, were obtained from Harlan/Timco, Houston, TX, USA.

Purification of CT, LT, and LT-B

CT was prepared as described by Mekalanos *et al.*²⁴. The culture conditions and purification of LT and LT-B were as previously described⁵. Organisms were cultured overnight at 37°C with vigorous aeration and agitation after inoculation with 10^6 viable bacteria per ml. The bacteria were harvested by centrifugation at 4°C, and the cells were suspended in TEAN buffer (0.05 M Tris, 0.001 M EDTA, 0.003 M NaN_3 , 0.2 M NaCl, pH 7.5)²⁵ and lysed using a French pressure cell. The crude lysate was then dialysed against TEAN buffer and, after centrifugation, applied directly to columns of Sepharose 4B (Sigma Chemical Co., St. Louis, MI, USA) equilibrated with TEAN buffer. LT or LT-B was then eluted from the columns with 0.2 M galactose in TEAN. Purified LT and LT-B were examined and found to be free of contaminating endotoxin with the *Limulus* amoebocyte lysate assay (Sigma Chemical Co.).

Immunization

The procedures for immunization were essentially the same as those described by Elson and Ealding¹⁰ for studying the influence of CT on induction of oral tolerance. Ovalbumin for immunization was Calbio-

bio (Calbiochem, La Jolla, CA, USA). Oral inoculation consisted of 0.5 ml PBS (0.01 M Na_2HPO_4 , 0.01 M KH_2PO_4 , 0.1 M NaCl, pH 7.2), 0.5 ml PBS containing 5 mg OVA, or 0.5 ml PBS containing 5 mg OVA, 25 μ g LT. For some experiments, LT was replaced by 25 μ g CT or with an equimolar amount of LT (17 μ g); for others, BSA was included with the LT. Following the oral inoculations, animals were bled *i.p.* with 1 μ g OVA in 20% Maalox (William H. Rorer Inc., Washington, PA, USA) or, where indicated, 1 μ g OVA in combination with 1 μ g BSA or 25 μ g LT in 20% Maalox. One week after the *i.p.* inoculation, animals were killed and assayed for serum IgG and, where indicated, mucosal IgA antibodies directed against OVA, BSA, and LT by ELISA. The number of animals in each immunization group is included in the legend to each figure.

Antibody assay

Animals were bled prior to euthanasia and sera were stored at -20°C until assayed. The small intestine from duodenum to ileal-cecal junction was excised, homogenized in a solution containing 50 mM EDTA and 0.1 mg ml⁻¹ of soybean trypsin inhibitor (Sigma Chemical Co.). Samples were homogenized with Tekmar Tissuemizer, clarified by centrifugation, filtered, resuspended in 1 ml TEAN buffer, dialysed against TEAN buffer, adjusted to a constant volume and stored at -20°C until assayed.

ELISA

Reagents and antisera for the ELISA were obtained from Sigma Chemical Co. Samples for ELISA were serially diluted in phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-TWEEN). For anti-LT determinations, microtitre plates were precoated with 1.5 μ g per well of mixed gangliosides (type III), then with 1 μ g per well of purified LT-B. Anti-OVA and anti-BSA were determined on microtitre plates precoated with OVA (10 μ g per well) or BSA (10 μ g per well), respectively. Serum anti-LT, anti-OVA and anti-BSA were determined with rabbit antiserum against mouse IgG conjugated to alkaline phosphatase. Mucosal anti-LT, anti-OVA and anti-BSA IgA were assayed with goat antiserum against mouse IgA (alpha-chain specific) followed by rabbit antiserum against goat IgG conjugated to alkaline phosphatase. Reactions were stopped with 3 M NaOH. Values for IgG and IgA were determined from a standard curve with purified mouse myeloma proteins (MOPC 315, $\gamma A(IgA\lambda 2)$; MOPC $\gamma G1$; Litton Bionetics, Inc., Charleston, SC, USA). Crossreactivity was determined by crossing reagent described²⁶. Mucosal IgA values are further corrected for contamination of mucosa with serum: Corrected mucosal IgA = mucosal IgA - [serum IgA X (mucosal IgG/serum IgG)].

Statistical analysis

The standard error of the mean was calculated for each data, and means of variously immunized groups were

compared by the Student *t* test. Statistical significance was considered to be $p \leq 0.05$.

Results

Effect of LT on oral tolerance to OVA (Table 1)

Four groups of Balb/c mice were immunized in this preliminary experiment. On day 0, each group was immunized orally as follows: group A received 0.5 ml PBS, group B received 0.5 ml PBS containing 5 mg OVA, group C received 0.5 ml PBS containing 5 mg OVA and 25 μ g LT and group D received 0.5 ml PBS containing 5 mg OVA and 17 μ g LT-B. Each regimen was administered again on days 7 and 14. On day 21, all animals were boosted i.p. with 1 μ g OVA in 20% Maalox.

As seen in Figure 1, animals primed orally with OVA developed a significantly lower serum IgG anti-OVA response following subsequent parenteral immunization with OVA than those primed with PBS alone and subsequently immunized parenterally with OVA (Figure 1B, 35 μ g ml⁻¹ versus Figure 1A, 95 μ g ml⁻¹). On the other hand, animals primed orally with OVA and LT developed a significantly higher serum IgG anti-OVA response than those in either of the other two groups (Figure 1C, 3194 μ g ml⁻¹). It was observed that the simultaneous administration of LT with OVA not only prevented the induction of tolerance to OVA caused by oral feeding of OVA alone, but also acted as an adjuvant for the antigen, increasing the anti-OVA IgG response by ≈ 90 -fold over the response seen in

Table 2 Effect of varying the timing and route of delivery of LT

Prime p.o.	Boost i.p.	IgG (μ g ml ⁻¹) ^a	IgA (ng ml ⁻¹) ^b
PBS 3X	OVA	16 \pm 3.9	ND
OVA 3X	OVA	11 \pm 1.6	11 \pm 11
OVALT 3X	OVA	6029 \pm 921	134 \pm 33
OVA 1X	OVA	2163 \pm 530	ND
OVALT 2X			
OVA 2X	OVA	32 \pm 16.9	ND
OVALT 1X			
OVA 3X ^c	OVA	11 \pm 4.5	ND
LT 3X			
LT 3X ^d	OVA	24 \pm 4.8	10 \pm 6
OVA 3X			
OVA 3X	OVALT	16 \pm 3.9	ND
OVALT 3X	OVALT	6029 \pm 793	53 \pm 39
OVALT 1X	OVA	4489 \pm 1339	154 \pm 94

ND, none detected

^aMean \pm s.e.m. as determined by ELISA. See text for details.

^bMean \pm s.e.m. as determined by ELISA. See text for details.

^cThis group received 0.5 ml PBS containing 5 mg OVA on days 0, 7, and 14, and received 0.5 ml PBS containing 25 μ g LT on days 1, 8, and 15.

^dThis group received 0.5 ml PBS containing 25 μ g LT on days 0, 7, and 14, and received 0.5 ml PBS containing 5 mg OVA on days 1, 8, and 15.

animals fed OVA alone (tolerant) and ≈ 30 -fold over the response seen in animals fed only PBS prior to the i.p. immunization with OVA.

We also wanted to determine if the observed response was a function of the binding component of the molecule (LT-B) or of the enzymatically active subunit A. We were in a unique position to make this determination since LT-B derived from the holotoxin by dissociation chromatography is invariably contaminated with residual traces of LT, whereas our LT-B recombinant clone produces LT-B free of any contaminating subunit A⁸. Therefore, in place of LT we substituted an equimolar amount of LT-B (17 μ g versus 25 μ g). Also seen in Figure 1, there was no significant difference between the group primed orally with OVA and LT-B (Figure 1D, 40 μ g ml⁻¹) and that primed with OVA alone (Figure 1B, 35 μ g ml⁻¹). Additional experiments failed to demonstrate any effect of LT-B on the induction of tolerance with levels of LT-B up to 100 μ g, the highest amount tested (data not shown). These experiments suggested that the ability to abrogate the induction of tolerance is a function of the A subunit of LT, since LT-B alone was unable to influence tolerance induction. Presumably, the B subunit is required to facilitate penetration of the A subunit into the cell.

Effect of varying the timing and route of delivery of LT (Table 2)

For this experiment, five groups of Balb/c mice were immunized as above. On day 0, each group was immunized orally as follows: group A received 0.5 ml PBS, group B received 0.5 ml PBS containing 5 mg OVA and group C received 0.5 ml PBS containing 5 mg of OVA and 25 μ g LT. This regimen was administered again on days 7 and 14. Group D received 0.5 ml PBS containing 5 mg OVA on day 0 and 0.5 ml PBS containing 5 mg OVA and 25 μ g LT on days 7 and 14. Group E received 0.5 ml PBS containing 5 mg OVA on days 0 and 7 and 0.5 ml PBS containing 5 mg OVA and 25 μ g LT on day 14. On day 21, animals in groups A-E were boosted i.p. with 1 μ g OVA in 20% Maalox.

As seen in Figure 2, animals fed LT with OVA after a single initial OVA prime (Figure 2D, 2186 μ g ml⁻¹)

Table 1 Effect of LT on oral tolerance to OVA

Prime p.o.	Boost i.p.	IgG (μ g ml ⁻¹) ^a	IgA ^b
PBS 3X	OVA	95 \pm 75	-
OVA 3X	OVA	35 \pm 4	-
OVALT 3X	OVA	3194 \pm 2150	-
OVALT-B 3X	OVA	40 \pm 7	-

^aMean \pm s.e.m. as determined by ELISA. See text for details.

^bNot determined

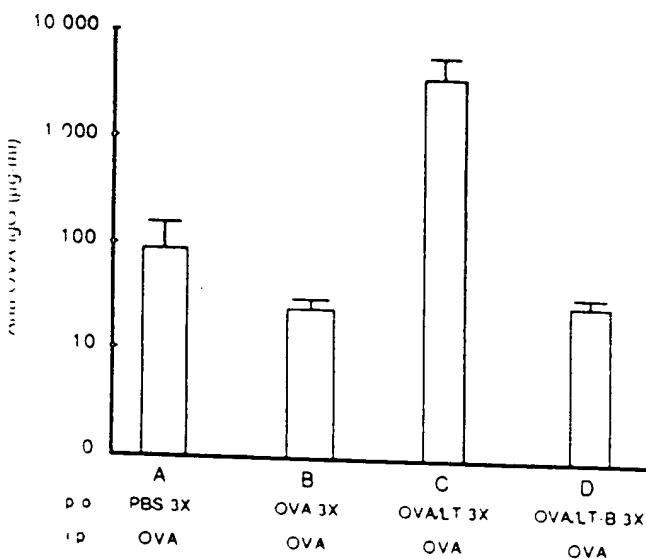


Figure 1 Effect of LT on oral tolerance to OVA. Mice were primed orally with PBS, OVA, or OVA in combination with LT or LT-B at weekly intervals as indicated. Animals were boosted i.p. with OVA and serum anti-OVA IgG was determined by ELISA. Bars represent mean \pm s.e.m. antibody response in each group 1 week after boost. Each group contained 5 mice.

ml⁻¹). This outcome could have resulted from either a timing-to-event phenomenon reflecting the shorter timing of LT administration prior to i.p. administration of the antigen or, conversely, could have represented a decreased effectiveness resulting from prior immunological experience with the antigen (see below). The effect of LT administration on the development of mucosal IgA was also determined (Figure 3). There was a significant increase in mucosal IgA anti-OVA (Figure 3C, 134 ng ml⁻¹) when LT was administered with the OVA during each of the three oral priming events. However, if the animals were immunized with OVA first, subsequent administration of LT with OVA failed to produce a detectable mucosal IgA anti-OVA response (Figure 3D,E).

for the study. Group F received 0.5 ml PBS containing 5 mg OVA on days 0, 7 and 14, and 0.5 ml PBS containing 25 µg LT on days 1, 8, and 15. Group G received 0.5 ml PBS containing 25 µg LT on days 0 and 14, and 0.5 ml PBS containing 5 mg OVA on days 1, 8, and 15. As above, animals in both groups were boosted i.p. with 1 µg OVA in 20% Maalox on day 16. Animals receiving LT before each administration of OVA had a slight but significantly higher level of serum IgG (Figure 2G, 24 µg ml⁻¹) anti-OVA than those orally primed with PBS alone (Figure 2A, 16 µg ml⁻¹) or those orally primed with PBS containing OVA (Figure 2B, 11 µg ml⁻¹) or those orally primed with OVA 1 day before each administration of LT (Figure 2F, 11 µg ml⁻¹). The mucosal anti-OVA IgA response in animals

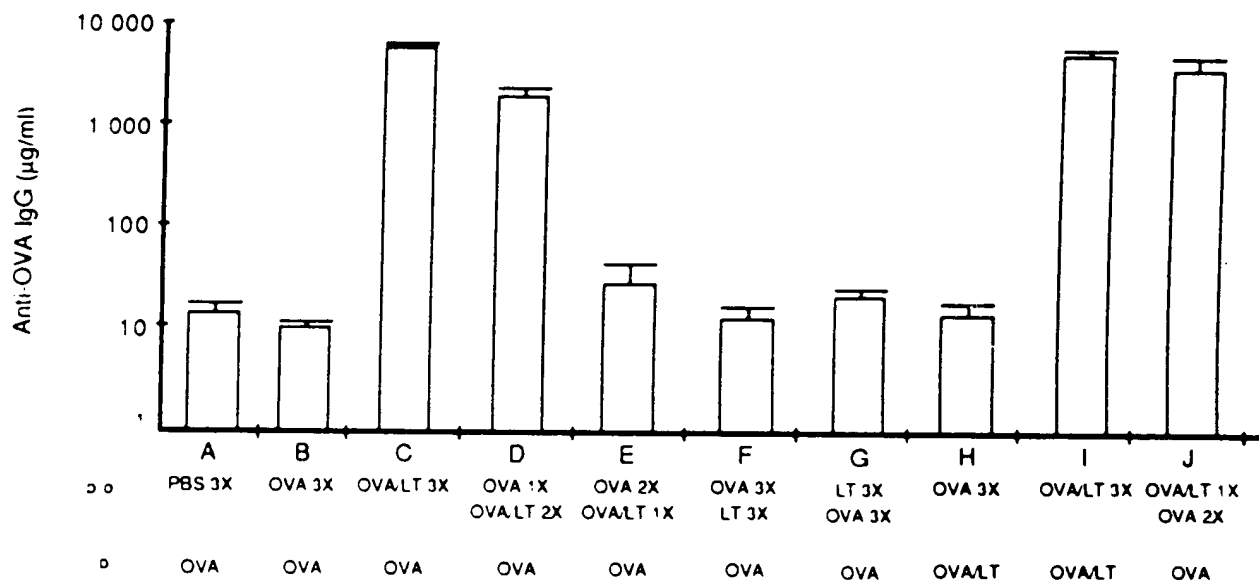


Figure 2 Effect of varying the timing and route of delivery of LT on anti-OVA serum IgG response. Mice were primed orally with PBS, OVA, or OVA in combination with LT at weekly intervals as indicated. Animals were boosted i.p. with OVA or OVA in combination with LT and serum anti-OVA IgG was determined by ELISA. Bars represent mean \pm s.e.m. antibody response in each group 1 week after boost. Each group contained five to seven mice. Data for this figure are presented in Table 2

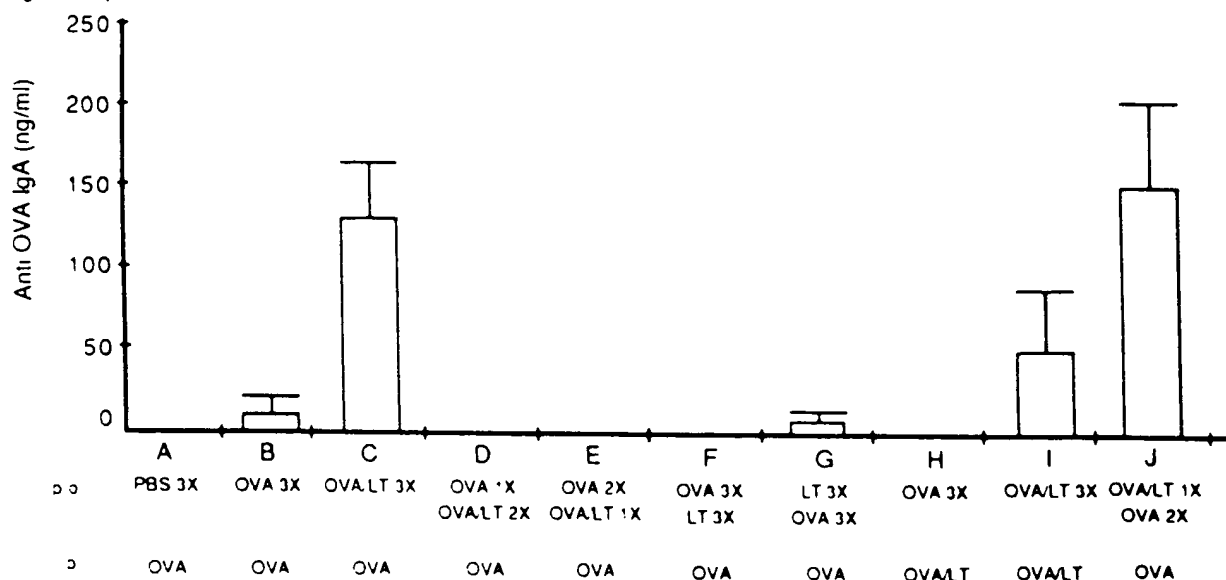


Figure 3 Effect of varying the timing and route of delivery of LT on anti-OVA mucosal IgA response. Mice were primed orally with PBS, OVA, or OVA in combination with LT at weekly intervals as indicated. Animals were boosted i.p. with OVA or OVA in combination with LT and mucosal anti-OVA IgA was determined by ELISA. Bars represent mean \pm s.e.m. antibody response in each group 1 week after boost. Each group contained five to seven mice. Data for this figure are presented in Table 2

those orally primed with PBS containing OVA (Figure 4B, $28 \mu\text{g ml}^{-1}$).

The mucosal anti-OVA IgA responses in these groups were also examined. As seen previously, mucosal anti-OVA IgA was present in detectable quantities only when OVA was administered with LT (Figure 5C and D) and the response was greater with the extended time between oral feeding with OVA/LT and i.p. boost with OVA. It is important to note that IgA values in this six week protocol were enhanced compared to the previous three week protocol ($\mu\text{g ml}^{-1}$ cf. ng ml^{-1}), possibly a reflection of the prolonged period of feeding. The fact that the anti-OVA response was greatly increased with the simultaneous administration of LT raised the possibility that LT could be used as an adjuvant for oral immunization to produce both a serum IgG and mucosal IgA response directed against the determinants of virulence of infectious agents.

Use of LT as an adjuvant with two unrelated antigens (Table 4)

In order to test further the potential of LT as an orally administered adjuvant, three groups of mice were immunized as follows. On day 0, group A received 0.5 ml PBS, group B received 0.5 ml PBS containing 5 mg OVA and 5 mg BSA and group C received 0.5 ml PBS containing 5 mg OVA, 5 mg BSA and $25 \mu\text{g}$ LT. This regimen was administered again on days 7 and 14. On day 21, each group of animals was boosted i.p. with $1 \mu\text{g}$ OVA and $1 \mu\text{g}$ BSA in 20% Maalox. Serum IgG and mucosal IgA responses were determined for both OVA and BSA. As seen in Figure 6, simultaneous administration of LT with OVA and BSA increased the serum IgG response to OVA ≈ 35 -

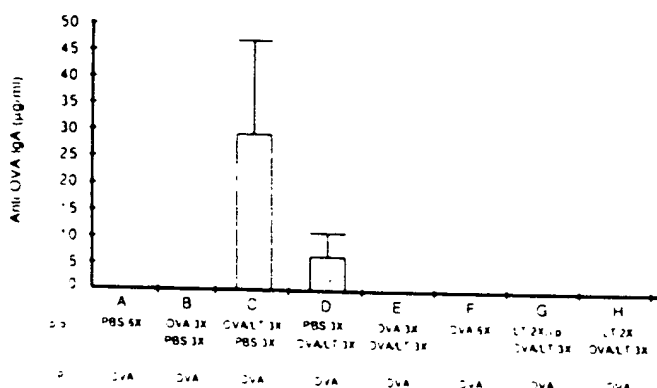


Figure 5 Effect of prior exposure to OVA on the ability of LT to influence anti-OVA mucosal IgA response. Mice were primed orally with PBS, OVA, or OVA in combination with LT at weekly intervals as indicated. Animals were boosted i.p. with OVA and mucosal anti-OVA IgA was determined by ELISA. Bars represent mean \pm s.e.m. antibody response in each group 1 week after boost. Each group contained five to eleven mice. Data for this figure are presented in Table 3

Table 4 Use of LT as an adjuvant with two unrelated antigens

Prime p.o.	Boost i.p.	IgG ($\mu\text{g ml}^{-1}$) ^a		IgA (ng ml^{-1}) ^b	
		anti-OVA	anti-BSA	anti-OVA	anti-BSA
PBS 3X	OVA/BSA	ND	ND	ND	334 \pm 81
OVA/BSA 3X	OVA/BSA	302 \pm 182	1 035 \pm 594	ND	422 \pm 180
OVA/BSA/LT 3X	OVA/BSA	10 710 \pm 4809	11 348 \pm 4273	814 \pm 282	385 \pm 83

ND: none detected

fold (Figure 6 upper left panel: B, $302 \mu\text{g ml}^{-1}$ versus $10 710 \mu\text{g ml}^{-1}$) and increased the serum anti-BSA response ≈ 11 -fold (Figure 6 upper right panel: $1035 \mu\text{g ml}^{-1}$ versus C, $11 348 \mu\text{g ml}^{-1}$). A mucosal anti-OVA IgA response was only detected in animals receiving LT with the oral immunization (Figure 6 lower left panel: C, 814 ng ml^{-1}). There was no significant change in mucosal anti-BSA IgA following the regimen (Figure 6 lower right panel), although

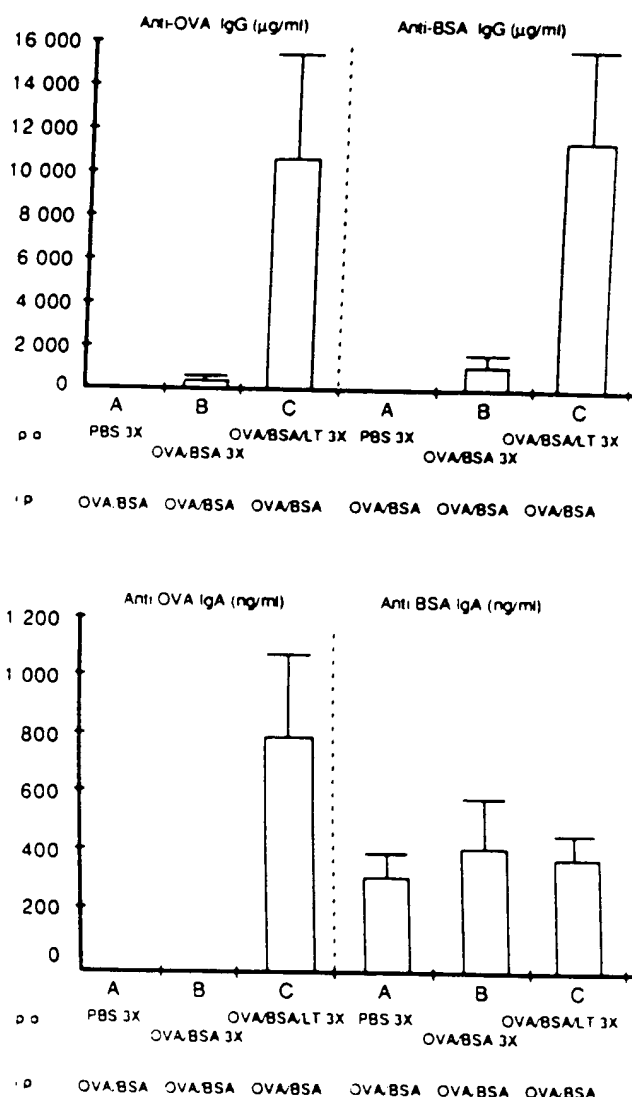


Figure 6 Use of LT as an adjuvant with two unrelated antigens. Mice were primed orally with PBS, OVA and BSA, or OVA and BSA in combination with LT at weekly intervals as indicated. Animals were boosted i.p. with OVA and BSA and serum anti-OVA IgG (upper left panel), serum anti-BSA IgG (upper right panel), mucosal anti-OVA IgA (lower left panel), and mucosal anti-BSA IgA (lower right panel) were determined by ELISA. Bars represent mean \pm s.e.m. antibody response in each group 1 week after boost. Each group contained six to ten mice. Data for this figure are presented in Table 4

increased response. Interestingly, there was no detectable anti-OVA or anti-BSA serum IgG response when OVA and BSA were administered i.p. following p.o. priming with PBS alone. This was different to the response observed with p.o. administration of OVA alone following PBS priming and may reflect the consequence of administering the combined antigens. Specifically, our experience indicates that prior exposure to an antigen in the absence of LT reduces or eliminates the subsequent ability of LT to influence the antibody response to that antigen when administered orally. Most commercial laboratory rodent foods are contaminated with BSA and this may have influenced the observed results.

Effect of route of immunization on anti-OVA responses (Table 5)

For this experiment, four groups of Balb/c mice were immunized. On day 0, each group was immunized orally as follows: all groups received 0.5 ml PBS containing 5 mg OVA and 25 µg LT. This regimen was administered again on days 7 and 14. On day 21, animals in group A received a subcutaneous (s.c.) boost with 1 µg OVA in 20% Maalox; group B was boosted intramuscularly (i.m.) with 1 µg OVA in 20% Maalox; group C was boosted i.p. with 1 µg OVA in 20% Maalox; and group D was not boosted.

As seen in the upper panel of Figure 7, groups of mice boosted i.p. with OVA developed significantly higher levels of serum IgG anti-OVA (Figure 7C, 4668 µg ml⁻¹) than those boosted either s.c. (Figure 7A, 2829 µg ml⁻¹) or i.m. (Figure 7B, 877 µg ml⁻¹) with OVA. The differences in serum anti-OVA IgG between groups boosted s.c. with OVA and control values for animals primed with PBS or with OVA alone and boosted i.p. with OVA were not statistically significant, while the differences in serum anti-OVA IgG between groups boosted i.m. with OVA and control values for animals primed with PBS or with OVA alone and boosted i.p. with OVA were statistically significant (not shown). The mucosal anti-OVA IgA responses in animals boosted by any of the three routes were not statistically significantly different from one another (Figure 7 lower panel). Importantly, animals that were not boosted (Figure 7D) failed to develop a significant serum IgG anti-OVA response (Figure 7D upper panel: 105 µg ml⁻¹) but did develop a significant mucosal IgA anti-OVA response (Figure 7D lower panel: 1846 ng ml⁻¹).

Discussion

It is well known that oral immunization can lead to the induction of systemic antigen-specific tolerance in

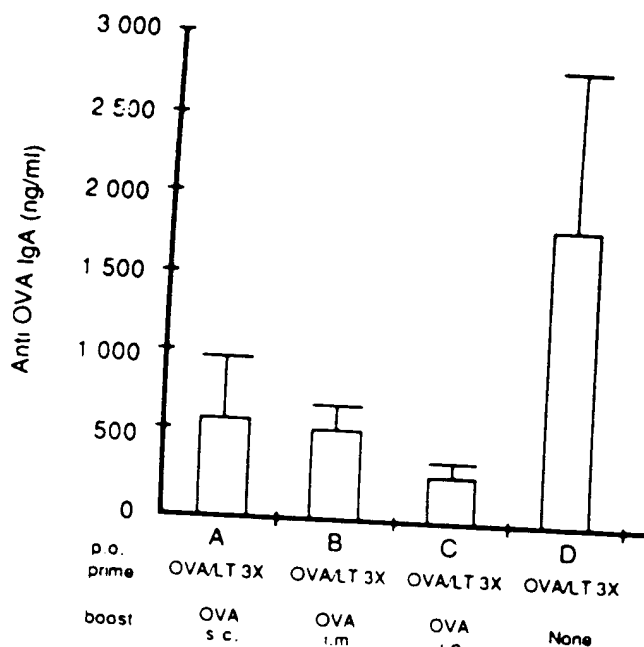
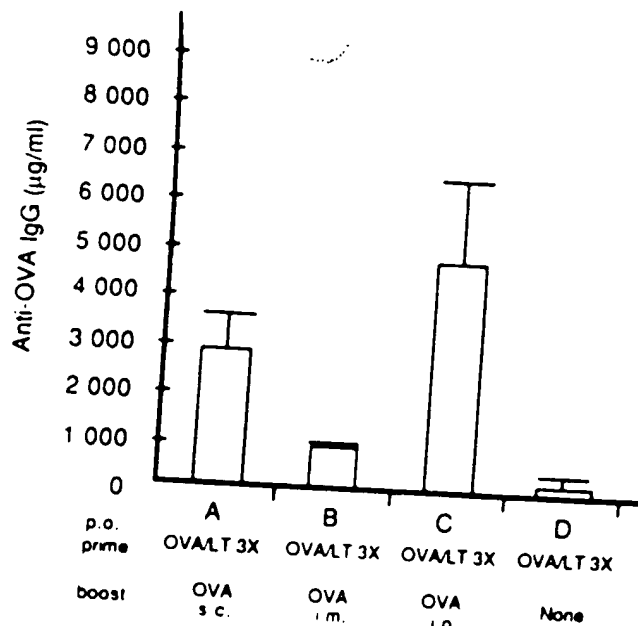


Figure 7 Effect of route of immunization on anti-OVA responses. Mice were primed orally with OVA in combination with LT at weekly intervals as indicated. Animals were boosted i.p., i.m., or s.c. with OVA or not boosted, and mucosal anti-OVA IgG and IgA were determined by ELISA. Bars represent mean ± s.e.m. antibody response in each group 1 week after boost. Each group contained five to six mice. Data for this figure are presented in Table 5

Table 5 Effect of route of immunization on anti-OVA responses

Time p.o.	Boost	IgG (µg ml ⁻¹) ^a	IgA (ng ml ⁻¹) ^b
/ALT 3X	OVA (s.c.)	2829 ± 900	614 ± 386
/ALT 3X	OVA (i.m.)	877 ± 245	514 ± 210
/ALT 3X	OVA (i.p.)	4668 ± 1831	302 ± 100
/ALT 3X	None	105 ± 58	1846 ± 974

^amean ± s.e.m. as determined by ELISA. See text for details
^bmean ± s.e.m. as determined by ELISA. See text for details

response to subsequent parenteral injection of the antigen¹. A variety of mechanisms has been proposed for this effect, including (a) antigen overload, (b) induction of antigen-specific suppressor T cells, and (c) clonal deletion of antigen-specific T and B cells (recently reviewed by Siskind²⁷). The abrogation of tolerance (or prevention of its induction) has also been widely studied²⁸⁻³⁰. In general, it has been observed that the ability to influence induction of tolerance depends upon the cellular basis of the state of tolerance.

The study reported here examined the ability of the heat-labile enterotoxin (LT) of *E. coli* to influence the induction and maintenance of tolerance in animals

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Principles of Gene Manipulation

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Mapping Peptide-binding Domains of the Substance P (NK-1) Receptor from P388D₁ Cells with Photolabile Agonists*

(Received for publication, September 9, 1994)

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The tachykinin substance P (SP) is a peptide transmitter of primary afferents. Its actions on both central and peripheral targets are mediated by a G-protein-coupled receptor of known primary structure. To identify contact sites between the undecapeptide SP and its receptor, we prepared radiolabeled photoreactive analogs of SP (H-RPKPQQFFGLM-NH₂) by replacing amino acids in the peptide with *p*-benzoyl-L-phenylalanine (BPA). SP, BPA³-SP, and BPA⁸-SP bind with high affinity ($K_d < 3$ nM) to SP receptors on the murine cell line P388D₁, triggering intracellular calcium responses. Both binding and calcium responses are blocked by the specific SP receptor antagonist CP-96345. On photolysis, radioiodinated BPA³-SP and BPA⁸-SP covalently label a heterogeneously glycosylated protein of about 75 kDa; labeling is abolished by excess unlabeled SP or CP-96345. The labeled receptors were digested with V8 protease and/or trypsin, and the resulting fragments were analyzed by electrophoresis, high pressure liquid chromatography, and chemical or enzymatic modification. BPA³-SP and BPA⁸-SP photoincorporate into different regions of the murine SP receptor. The results establish that the third and the eighth positions of SP, respectively, interact with the NH₂-terminal extracellular tail (residues 1–21) and second extracellular loop (residues 173–183) of the SP receptor. A model for the agonist peptide-binding sites of the SP receptor is proposed based on photoaffinity labeling and mutagenesis studies.

A large majority of the known receptors belong to the G-protein-coupled receptor superfamily (Baldwin, 1994). These receptors are characterized by the presence of seven hydrophobic regions of primary structure thought to represent transmembrane domains. The receptors lie in the bilayer such that the amino-terminal region of the protein is extracellular and the carboxyl-terminal region is cytoplasmic. The agonists which bind to and activate G-protein-coupled receptors vary widely in size, from glycoprotein hormones (>30 kDa) to single photons. The larger agonists (>10 kDa; e.g. thyroid stimulating hormone and follicle stimulating hormone) bind to the amino-terminal region of their G-protein-coupled receptors, while the smaller agonists (<0.2 kDa; e.g. norepinephrine, serotonin, and photons) bind within the plane of the bilayer between the seven

transmembrane domains (Bockaert, 1991). Essentially all characterized receptors for bioactive peptides (0.5–5 kDa) are also members of the G-protein-coupled receptor superfamily, but which regions of their receptors interact with these agonists of intermediate size has not yet been defined.

The undecapeptide substance P (SP)¹ has been identified as a neurotransmitter associated with pain modulation and neurogenic inflammation (Pernow, 1983; Otsuka and Yoshioka, 1993). SP belongs to the tachykinin peptide family which is characterized by a conserved COOH-terminal sequence -FXGLM-NH₂, where X is an aromatic or aliphatic amino acid (Maggio, 1988). The SP receptor (also known as the neurokinin-1 or NK-1 receptor) has been cloned from several species including human, mouse, rat, and guinea pig and displays a very high degree of primary sequence homology across species (Gerard *et al.*, 1993). The SP receptor (SPR) is a member of the G-protein-coupled receptor superfamily, as are receptors for other peptides in the tachykinin family.

Chimeric and point-mutated SP receptors have been constructed to probe receptor structure-function in an attempt to identify binding domains for peptide agonists and nonpeptide antagonists as well as domains associated with agonist-stimulated second messenger responses (e.g. Cascieri *et al.*, 1994; Fong *et al.*, 1992a, 1992b, 1993, 1994a, 1994b; Gether *et al.*, 1993a, 1993b, 1993c, 1994; Huang *et al.*, 1994a, 1994b; Jensen *et al.*, 1994; Sachais *et al.*, 1993; Yokota *et al.*, 1992; Zoffmann *et al.*, 1993). These studies have indicated that both the extracellular and transmembrane domains of the SP receptor are important for the binding of agonist, and several specific residues conserved in all species examined have been identified as important for peptide binding. Analysis of SP analogs further suggested the COOH-terminal carboxamide of SP may interact with residues in the second transmembrane domain (Huang *et al.*, 1994b). However, the identification of a particular residue as necessary for agonist binding does not necessarily imply direct interaction of that side chain with agonist, as loss of function may instead result from changes in protein folding. Since the binding of SP (1350 Da) must involve a larger number of receptor/ligand contacts than small nonpeptide agonists (e.g. norepinephrine, 170 Da), it has not been possible to define the interaction of SP and its receptor by mutagenesis alone.

Photoaffinity labeling has been proven to be a useful tool in identifying structural domains of receptors involved in ligand

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¹ The abbreviations used are: SP, substance P; BPA, *p*-benzoyl-L-phenylalanine; BPA³-SP, Y³BPA³-SP; BPA⁸-SP, Y⁸BPA⁸-SP; CP-96345, (2S,3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-aza-bicyclo[2.2.2]octan-3-amine; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); Endo F, *F. meningosepticum* endoglycosidase F; FCS, fetal calf serum; Fmoc, fluorenyl-methoxycarbonyl; HPLC, high performance liquid chromatography; NK-1R, neurokinin-1 receptor (same as SPR); PAGE, polyacrylamide gel electrophoresis; SPR, substance P receptor (same as NK-1R); TM, transmembrane; V8, *S. aureus* V8 protease; cpm, counts/minute.

binding (e.g. Dohlman *et al.*, 1991). This technique offers a unique approach by directly identifying the contact regions of a receptor and its ligands. As an essential complement to mutagenesis approaches, we have applied photoaffinity labeling to identify agonist peptide binding domains of the SP receptor. *p*-Benzoyl-L-phenylalanine (BPA), a photoreactive amino acid, has been used to replace amino acids in peptides for receptor photoaffinity labeling (Dorman and Prestwich, 1994). Photoactivated (triplet biradical) BPA reacts preferentially with C-H bonds but has low reactivity toward water; furthermore, the chromophore can be activated in the visible, avoiding protein-damaging UV wavelengths. In previous work by others (Boyd *et al.*, 1991a, 1991b, 1994; Kage *et al.*, 1993), an SP derivative containing BPA at position 8 and acylated with 3-(3-iodo-4-hydroxyphenyl) propionic acid at the side chain of Lys³ has been synthesized to study the SP receptor. Photolysis of this ligand with membrane-bound SP receptors from rat submaxillary gland led to about 70% incorporation of bound label into two polypeptides (46 and 53 kDa); enzymatic studies suggested that the smaller protein resulted from proteolysis of the larger (Kage *et al.*, 1993).

P388D₁ cells, a nontransfected murine macrophage/monocyte cell line (Dawe and Potter, 1957), express a high density of functional SP receptors (Persico *et al.*, 1988; Li *et al.*, 1994) but no detectable levels of other tachykinin receptors.² The SP receptors of this cell line are coupled to Ca²⁺ mobilization (Li *et al.*, 1994). In the present study we have used two site-specific, high affinity photolabile analogs of SP (incorporating BPA in the third (BPA³) or eighth (BPA⁸) position) to label the SP receptor of P388D₁ cells and map the peptide-binding domains of the receptor for each ligand.

EXPERIMENTAL PROCEDURES

Preparation of Fluoren-9-ylmethoxycarbonyl-4-benzoylphenylalanine (Fmoc-BPA)—Racemic DL-BPA was synthesized and resolved into L- and D-BPA as described by Kauer *et al.* (1986). The resolved amino acid (or alternatively the racemic mixture) was treated directly with Fmoc-chloroformate (Aldrich) or Fmoc-hydroxysuccinimide (Sigma) to provide the protected amino acid (Fmoc-BPA) for solid-phase synthesis.

Peptide Synthesis—Peptide synthesis of the SP analogs, BPA³-SP and BPA⁸-SP, was performed by our departmental Biopolymers Facility or by Quality Control Biochemicals (Hopkinton, MA) using a standard Fmoc solid-phase synthetic strategy (Maggio *et al.*, 1992). The crude synthetic peptide was then purified by reverse-phase high performance liquid chromatography (HPLC) using a C₁₈ column (Vydac 4.6 × 250 mm, 5 μm, 300 Å) on a Waters Liquid Chromatographic System equipped with a variable wavelength UV detector. The column was eluted with a linear water-acetonitrile gradient (26–56% acetonitrile, 1%/3 min; 1.0 ml/min) containing 10 mM trifluoroacetic acid. The racemic peptide had two major UV active (254 nm) components of equal intensity. The L-BPA peptide was identified by elution position; that is, L-BPA⁸-SP (synthesized from Fmoc-L-BPA) showed only one UV active peak which was coincident with the earlier eluting HPLC peak of DL-BPA⁸-SP (synthesized from Fmoc-DL-BPA). Thus, the earlier HPLC peak corresponds to L-BPA⁸-SP and the later peak to D-BPA⁸-SP. In addition, Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanineamide) formed an adduct (Marfey, 1984) with isolated L-BPA that precedes that formed from D-BPA. When the L-BPA⁸-SP and D-BPA⁸-SP fractions were tested for receptor binding and biological activity, the earlier eluting peptide, i.e. L-BPA⁸-SP, was much more active than the later eluting D-BPA⁸-SP analog. By analogy to the greater activity of L-Phe⁸-SP relative to D-Phe⁸-SP (Fournier *et al.*, 1982), this result further confirms that the first of the paired HPLC peaks is the L-BPA-isomer. The L-BPA diastereomer has also been shown to precede the D-BPA diastereomer of other peptides in reverse-phase HPLC elution (Shoelson *et al.*, 1993).

The isolated L-BPA⁸-SP was analyzed for purity and correct structure by amino acid analysis, laser desorption mass spectroscopy, and sequence. The peptide was sequentially Y_{0.2} R_{0.9} P_{0.7} K_{1.0} P_{0.7} Q_{1.0} Q_{1.0} F_{1.2} (BPA) G_{1.0} L_{1.0} M_{0.8} with *m/z* 1616.2 ((M+H)⁺). Neither BPA or its phenylthiohydantoin derivative elute from the analyzer column under

standard conditions (Kauer *et al.*, 1986). Nevertheless, the high UV extinction coefficient of BPA at 254 nm, $\epsilon = 21 \times 10^3$, verifies that BPA is incorporated (Kauer *et al.*, 1986), confirming the results of mass spectrometry. Tyrosine was partially destroyed under the hydrolysis conditions employed.

Preparation of Radioligands—The radioligand [¹²⁵I]BPA⁸-SP ([¹²⁵I]iodotyrosyl⁸-L-BPA⁸-SP) was formed using general peptide iodination techniques previously described (Too and Maggio, 1991). Typically 10 nmol of dry peptide was dissolved in 50 μl of 0.5 M sodium phosphate buffer, pH 7.5, and vortexed with 1 mCi of Na¹²⁵I (10 μl, Amersham Corp.). Chloramine-T (10 μg in 10 μl of water) was added to activate iodine incorporation, and Na₂S₂O₅ (100 μg in 10 μl of water) was added after 1 min of vortexing to quench the reaction. The mixture was diluted and acidified with 0.6 ml of 60 mM trifluoroacetic acid, and 25 μl of 2% bovine serum albumin was added to limit nonspecific adsorption. To separate the peptide from the unincorporated ¹²⁵I, the mixture was then applied to an activated C₁₈ Sep-Pak cartridge (Waters) and the iodide and peptide eluted with a series of 0.5-ml portions of 10 mM trifluoroacetic acid solutions of increasing alcohol (ethanol/methanol, 1:1) content, 10, 10, 20, 40, 60, 80, 90, 95, and 100%. Unincorporated iodide elutes immediately, while the peptide is retained until the alcohol concentration reaches about 60%. The peptide fractions (containing both oxidized and reduced methionine), eluting with 60–90% alcohol, were pooled and reduced in volume under a nitrogen stream. After the addition of 20% (v/v) β-mercaptoethanol, the sample was heated at 90 °C for 2 h to reduce methionine sulfoxide to its thioether. Further purification was achieved by reversed-phase HPLC on a Vydac C₁₈ column as above. The eluate was collected in fractions during gradient elution and the fractions counted for radioactivity. The reduced (Met) and moniodinated (Tyr) peptide eluted in a well-resolved peak (34.6% acetonitrile), predictably later than the original compound (33.2%) or the oxidized products, but prior to the diiodinated reduced peptide. The reduced moniodinated tracer (specific activity ~2000 C/mmol; 1C_i = 37 GBq) was protected from oxidation by 0.5% β-mercaptoethanol (v/v) added immediately after purification and stored at –20 °C until use. Radiiodinated BPA⁸-SP was prepared similarly.

Cell Culture—The murine cell line P388D₁ (Dawe and Potter, 1957; Persico *et al.*, 1988) was a gift of Dr. J. Jackie (Harvard Medical School) and has been maintained in our laboratory in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS).

Calcium Measurements—P388D₁ cells were cultured on 12-mm diameter round glass coverslips that had been pretreated with laminin. These were used within 24–48 h after plating. Dye loading was achieved by exposing the cells to fura-2 acetoxymethyl ester at a concentration of 8 μM for 30 min at room temperature. The cells were then washed with 2% bovine serum albumin and kept on ice until used. Experiments were performed using a standard saline buffer with the following components: NaCl 120 mM, KCl 4.2 mM, CaCl₂ 2.5 mM, MgSO₄ 1.0 mM, Na₂HPO₄ 1.0 mM, glucose 12 mM, and HEPES 10 mM, pH 7.40.

Fluorescence measurements were made using a Nikon microscope optically linked to a PTI Deltascan instrument (Photon Technologies) that produces dual excitation at 340 and 380 nm. Emitted light was collected after passing through a 510-nm band pass filter. A 40X Nikon fluor objective was used and the field was limited to about 15–20 cells for data collection.

Ligand Binding of P388D₁ Cells—P388D₁ cells (5 × 10⁵ cells/well) were inoculated on FCS precoated 24-well plates and cultured overnight. The confluent cells (~1 × 10⁶ cells/well) were washed twice (0.5 ml/well) with ice-cold buffer (Dulbecco's modified Eagle's medium + 20 mM HEPES, pH 7.2) and incubated with 0.5 ml of buffer on ice for at least 10 min. Then radioactive ligand, in the presence or absence of unlabeled displacers, was added to a final concentration of 150 pM (~5 × 10⁵ cpm/ml) and incubated for 2 h. Nonspecific binding is defined as binding in the presence of 10 μM unlabeled SP. After incubation, the cells were washed twice with 0.5 ml/well phosphate-buffered saline (104 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2), then solubilized by 0.5 ml of lysis buffer (1% Nonidet P-40, 0.2% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0) for 20 min and transferred for gamma counting. Unlabeled SP and CP-96345 were stored as 10 mM stock solutions in dimethyl sulfoxide. Dimethyl sulfoxide at less than 3% (v/v) had no detectable effects on the binding assay.

Photoaffinity Labeling of P388D₁ Cells—Cells (5 × 10⁶) were inoculated on FCS-pretreated dishes (60 mm) and grown for at least 12 h before labeling. The cultured cells (~1 × 10⁷) were washed twice (5 ml) with ice-cold buffer (Dulbecco's modified Eagle's medium + 20 mM HEPES, pH 7.2) and incubated with 5 ml of buffer on ice for at least 10 min. The photolabile radioligand, in the presence or absence of un-

² H.-P. Too and J. E. Maggio, unpublished results.

labeled displacers, was added to a final concentration of 2 nM ($\sim 6 \times 10^6$ cpm/ml) and incubated 2 h. The dishes were then irradiated on ice for 15 min using a focused HBO 100-watt mercury short arc lamp through an optical filter to eliminate light below 310 nm. A second filter removed infrared wavelengths to minimize sample heating during photolysis.

After photolysis, the cells were washed twice (5 ml) with phosphate-buffered saline and transferred to a microfuge tube to collect cell pellets by centrifugation at $16,000 \times g$ for 10 min. The pelleted cells were resuspended in 0.3 ml of 5 mM Tris-HCl, pH 8.0, and hypotonically lysed for 30 min at room temperature. Then the samples were homogenized and centrifuged at $500 \times g$ for 15 min to remove debris. The resulting supernatants were sedimented at $16,000 \times g$ for 30 min and the membrane pellets stored at -20°C until analysis. The presence or absence of a mixture of protease inhibitors (bacitracin, chymostatin, and leupeptin) did not affect the results of binding or photolysis experiments.

Partial Purification of the Labeled Complex—SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970) using 1.5-mm 8% gels. The labeled cell membranes were solubilized in $1 \times$ SDS sample buffer (10% glycerol, 5% 2-mercaptoethanol, 3% SDS, 0.025% bromophenyl blue, 125 mM Tris-HCl, pH 6.8) for 30 min at room temperature. After electrophoresis, the gel was directly dried on a filter paper and exposed to x-ray film (Kodak XAR-5) with an intensifying screen (DuPont). The labeled bands were isolated from the preparative gel using a passive elution protocol similar to that described by Blanton and Cohen (1994). After autoradiography, radioactive bands of the labeled complex were excised from dried gels and rehydrated with extraction buffer (0.1% SDS, 100 mM NH_4HCO_3 , pH 7.8). The gel slices were macerated and eluted for 1–4 days with extraction buffer. The eluted protein was filtered (Whatman No. 1) and concentrated using Centrprep-10 or Centricon-10 (Amicon). Finally, the labeled complex was precipitated by cold acetone (85–90%, v/v) overnight at -20°C . The precipitate was dried and stored at -20°C until use. For all electrophoretic gels, the ratio of bis-acrylamide to acrylamide was 3%. For gels above 12% acrylamide, 1% glycerol was added to the running buffer to prevent cracking of gels during drying.

Endoglycosidase F Digestion of the Partially Purified Complex—The acetone precipitate was resuspended in 10 mM EDTA, 0.1% SDS, 0.5% *N*-octylglucoside, 100 mM NH_4HCO_3 , pH 7.8, and then digested with *Flavobacterium meningosepticum* endoglycosidase F (Endo F) (Boehringer Mannheim) for 2 days at room temperature.

Protease Digestion of the Partial Purified Complex—The acetone precipitate was resuspended in 0.1% SDS, 100 mM NH_4HCO_3 , pH 7.8, and then digested with *Staphylococcus aureus* V8 protease (V8) (Boehringer Mannheim) for 2–4 days at room temperature or L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-treated bovine trypsin (Sigma) for 1–4 days at room temperature. Both BPA³-SP and BPA⁶-SP completely resist cleavage by these proteases under these conditions (not shown) as both ligands lack glutamic acid residues, and basic residues are protected by adjacent prolines (Fig. 1).

HPLC of Enzymatic Digests—The V8-digested samples were loaded into a Vydac C₈ column (2.1×150 mm, 5 μm , 300 Å) and eluted with increasing solvent B (0.09% trifluoroacetic acid in 60% acetonitrile, 40% 2-propanol) in solvent A (0.1% trifluoroacetic acid in water) at a flow rate of 0.25 ml/min. The elution of ¹²⁵I-labeled SPR fragments was monitored by gamma counting of the fractions. More than 90% of injected radioactivity was recovered in the eluate for all HPLC experiments.

5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) Modification—The HPLC fractions were dried and resuspended in 100 mM NH_4HCO_3 , pH 7.8. Dithiothreitol was added to a final concentration of 25 mM and the mixture incubated for 30 min at 50°C , then DTNB (final concentration 75 mM) was added and allowed to react with the peptide for at least 30 min. The mixture was then diluted with 20% solvent B and loaded onto reverse-phase-HPLC as described.

RESULTS

Specificity of BPA³-SP and BPA⁶-SP—Both BPA³-SP and BPA⁶-SP (Fig. 1) are full and potent agonists relative to SP for the calcium responses of P388D₁ cells. This action is completely blocked by CP-96345, a specific nonpeptide antagonist of the SP receptor (Fig. 2). Both photoreactive ligands bound to SPR (Table I) with the same affinity (IC_{50} values ~ 3 nM) as SP and the binding of each was similarly inhibited by CP-96345 (IC_{50} values ~ 35 nM).

Photoaffinity Labeling of SP Receptor—After photoinsertion of radioiodinated BPA³-SP or BPA⁶-SP bound to P388D₁ cells, two radioactive bands were observed on SDS-PAGE. A major

Agonist	Residue Number											
	0	1	2	3	4	5	6	7	8	9	10	11
SP		Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met-NH ₂
BPA ³ -SP	Tyr	Arg	Pro	BPA	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met-NH ₂
BPA ⁶ -SP	Tyr	Arg	Pro	Lys	Pro	Gln	Gln	Phe	BPA	Gly	Leu	Met-NH ₂

FIG. 1. Primary structures of SP, BPA³-SP, and BPA⁶-SP. Addition of Tyr at the NH₂ terminus facilitates radioiodination. Lys³ and Phe⁶ are respectively replaced by BPA to give BPA³-SP and BPA⁶-SP.

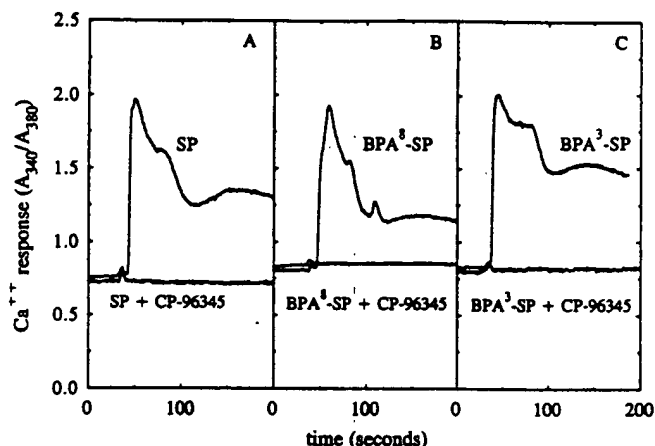


FIG. 2. SP, BPA³-SP, and BPA⁶-SP induce calcium responses in P388D₁ cells. $[\text{Ca}^{2+}]_{\text{free}}$ was measured with fura-2 using 12–18 cells in an optical field. Agonists (10 nM) were perfused over the cells for 30 s. CP-96345 (1 μM) was preincubated with the cells for 30 s before adding the mixture of agonist and CP-96345. Experiments were carried out at room temperature (22°C).

TABLE I
 IC_{50} values of substance P and CP-96345 for three ligands binding to P388D₁ cells

Ligands ^a	IC_{50} (nM) ^b	
	Substance P	CP-96345
[¹²⁵ I]BHSP	2.30 \pm 0.40	33.3 \pm 11.5
[¹²⁵ I]BPA ³ -SP	2.75 \pm 0.95	33.3 \pm 11.5
[¹²⁵ I]BPA ⁶ -SP	3.00 \pm 1.00	36.7 \pm 5.70

^a Final concentration 0.15 nM.

^b Mean \pm S.D. of at least three experiments.

broad band of 75 kDa accounted for about 95% of the radioactivity, while a minor band of 205 kDa accounted for about 5% (Fig. 3). Labeling of both bands was completely inhibited by SP (Fig. 3) or CP-96345 (not shown). For BPA⁶-SP, $46 \pm 3\%$ of bound ligand is recovered in the broad 75 kDa band; for BPA³-SP, the major band represents about 6% of the total bound radioactivity. For both ligands, deglycosylation with Endo F shifted the broad major band to a sharp band of 42 kDa (Fig. 4), indistinguishable from the molecular mass of the murine SPR calculated from its cDNA sequence (Sundelin *et al.*, 1992). The major band labeled by both photoprobes was partially purified by preparative SDS-PAGE and used for further studies. Incubation of cells with the photoprobes in the dark resulted in no detectable incorporation into protein.

V8 Digestion of the Labeled SP Receptor—V8 protease (Glu-C) cleaves proteins specifically at the COOH-terminal side of glutamate residues under the conditions employed. V8 digestion of partially purified [¹²⁵I]BPA⁶-SP-labeled SPR showed five detected bands (33, 25, 19, 9, and 3.2 kDa). The larger proteolytic fragments were converted into the smaller ones at higher concentration of V8 protease, with the smallest fragment, designated BPA⁶-SPR-3.2k, being the limit digest (Fig. 5A). Double digestion of [¹²⁵I]BPA⁶-SP-labeled SPR with Endo F and V8 protease revealed the same five-band pattern seen

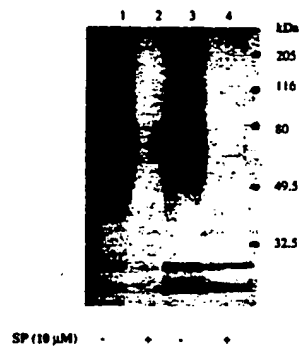


FIG. 3. Autoradiography of P388D, cell membranes photoaffinity labeled with $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ and $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ following SDS-PAGE (8% gel). Cell culture, photolysis, cell membrane preparation, and solubilization were as described under "Experimental Procedures." After electrophoresis, the gel was stained with 0.1% Coomassie Blue in $\text{MeOH}/\text{AcOH}/\text{H}_2\text{O}$ (4:1:5) and destained in the same solvent. The same amount of protein was found in each lane (not shown). Lanes 1 and 2, $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled membranes; lanes 3 and 4, $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled membranes. The labeling was carried out in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 μM unlabeled SP. Molecular weights are indicated at the right.

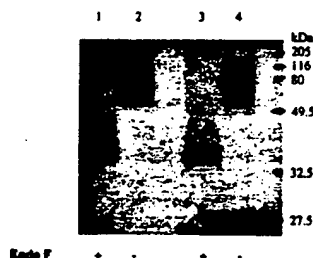


FIG. 4. Autoradiography of Endo F deglycosylated photolabeled SP receptor following SDS-PAGE (12% gel). Acetone-precipitated $[^{125}\text{I}]$ -labeled SPR (5000–8000 cpm) from preparative SDS-PAGE was dissolved in 0.1% SDS, 10 mM EDTA, 0.5% *N*-octylglycoside, 100 mM NH_4HCO_3 , pH 7.8, and digested by Endo F (0.8 unit) for 2 days at room temperature. The gel was directly dried without fixation or staining prior to autoradiography. Lanes 1 and 2, $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled SPR; lanes 3 and 4, $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled SPR. The samples were treated with (lanes 1 and 3) or without (lanes 2 and 4) Endo F. Molecular weights indicated are at the right.

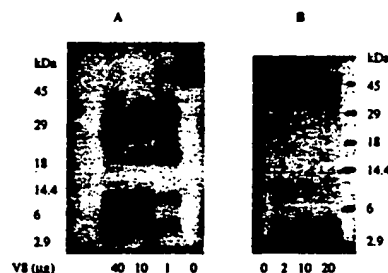


FIG. 5. Autoradiography of V8 protease digest of photolabeled SP receptor following SDS-PAGE. Acetone precipitated $[^{125}\text{I}]$ -labeled SPR (5000–8000 cpm) from preparative SDS-PAGE was dissolved in 0.1% SDS, 100 mM NH_4HCO_3 , pH 7.8, and digested by the indicated amount of V8 protease for 4 days at room temperature. The gel was directly dried for autoradiography. A, $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled SPR (17.5% gel); molecular weights are indicated at the left. B, $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled SPR (18% gel); molecular weights are indicated at the right.

with V8 digestion alone.

Peptide fragments from V8 digestion of $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled SPR were isolated by HPLC (Fig. 6A). One major peak (~33% solvent B), accounting for most of the eluted radioactivity, corresponded to $\text{BPA}^3\text{-SPR-3.2k}$ (Fig. 6B). $\text{BPA}^3\text{-SPR-3.2k}$ and $\text{BPA}^3\text{-SP}$ tracer eluted in a similar position on reverse-

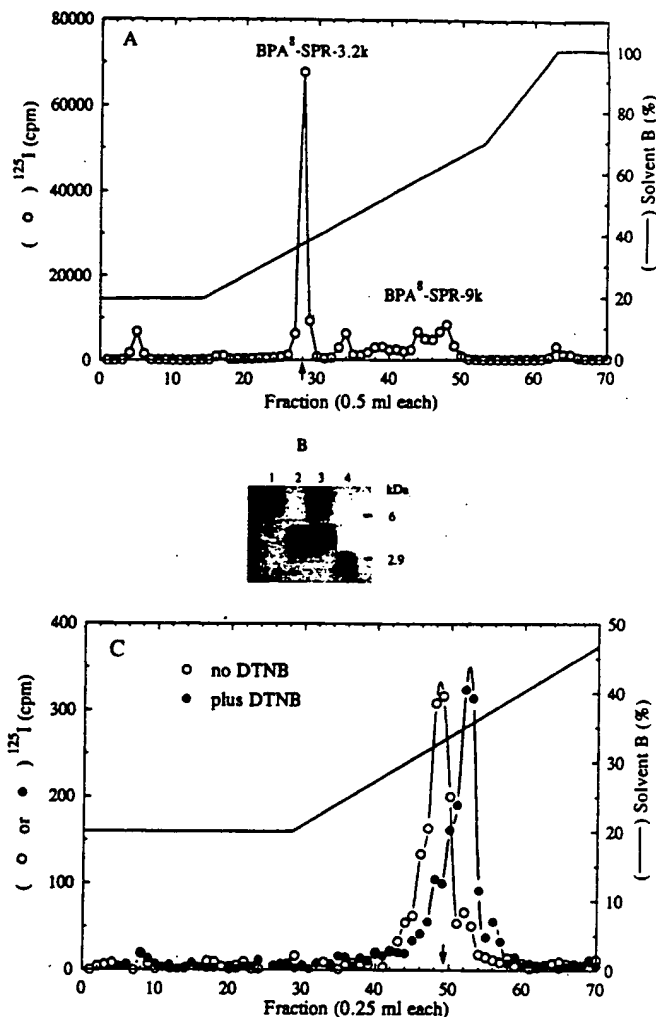


FIG. 6. Analysis of the $\text{BPA}^3\text{-SPR-3.2k}$ fragment. $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ -labeled SPR was digested by V8 protease as described in Fig. 5. The digests were separated by reverse-phase HPLC as described under "Experimental Procedures" (microbore C_8 column, organic phase 60% acetonitrile, 40% isopropanol). The solvent gradient (flow rate 0.25 ml/min) was as follows: 20–70% solvent B in 62.5 min; 70–100% solvent B in 15 min. Fractions (0.5 ml each) were counted for ^{125}I (A). The digests and HPLC fractions were dried and analyzed by SDS-PAGE on an 18% gel (B). Lane 1, minor HPLC peak, fraction 44–48; lane 2, major HPLC peak, fractions 27–29; lane 3, V8 digest prior to HPLC fractionation; lane 4, $[^{125}\text{I}]\text{BPA}^3\text{-SP}$; molecular weights are indicated at the right. $\text{BPA}^3\text{-SPR-3.2k}$ fractions were dried and treated with or without DTNB as described, then separated by HPLC using a solvent gradient of 20–50% solvent B in 37.5 min (C). Arrow indicates the elution position of $[^{125}\text{I}]\text{BPA}^3\text{-SP}$.

phase HPLC but were cleanly resolved by SDS-PAGE (Fig. 6B). A second peak of radioactivity eluting from the HPLC column, accounting for most of the recovered radioiodine not in $\text{BPA}^3\text{-SPR-3.2k}$, corresponded to $\text{BPA}^3\text{-SPR-9k}$. Further digestion of $\text{BPA}^3\text{-SPR-9k}$ with V8 protease converted this fragment to $\text{BPA}^3\text{-SPR-3.2k}$ (not shown).

$\text{BPA}^3\text{-SPR-3.2k}$, the limit digest, was reacted with DTNB, a specific sulfhydryl modification reagent which converts free peptidyl-SH groups to mixed disulfides of 2-nitro-5-thiobenzoic acid. Treatment of $\text{BPA}^3\text{-SPR-3.2k}$ with DTNB shifted the HPLC elution position of the peptide to later elution by 3.2% solvent B (Fig. 6C). A parallel sample incubated identically but without DTNB showed no change in elution position.

V8 digests of SPR photolabeled with $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ displayed a different pattern of proteolytic fragments than digests of SPR photolabeled with $[^{125}\text{I}]\text{BPA}^3\text{-SP}$ (Fig. 5). A major fragment of

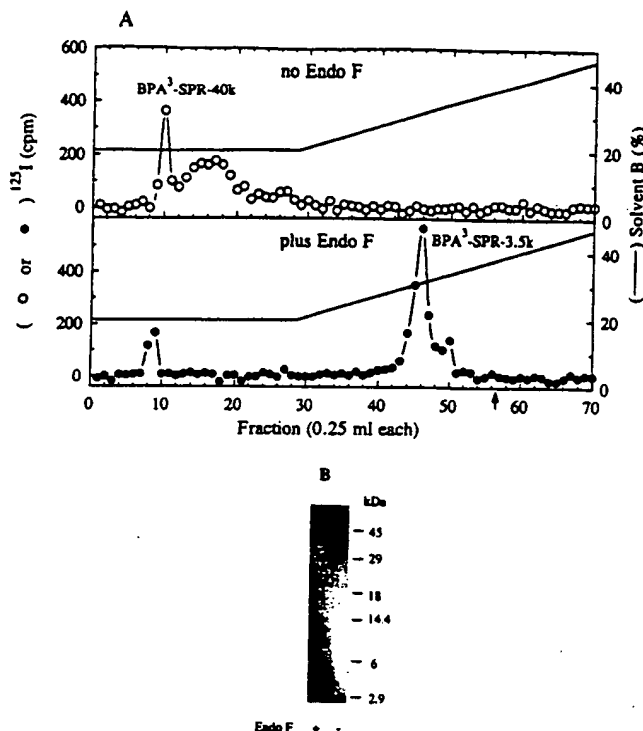


FIG. 7. Analysis of the BPA³-SPR-40K fragment. [¹²⁵I]BPA³-SP-labeled SPR was digested by V8 protease as described in Fig. 5. The digests were separated by HPLC as described in Fig. 6A. Fractions eluting at the void volume (unretained by the reverse-phase column) were dried and treated with or without Endo F. The samples then reanalyzed by HPLC (A) as described in Fig. 6C and by SDS-PAGE (B) on an 18% gel. Lane 1, HPLC fractions 43–48 after Endo F; lane 2, HPLC fractions 9–19 without Endo F; molecular weights are indicated at the right. Arrow (A) indicates the elution position of [¹²⁵I]BPA³-SP.

40 kDa (BPA³-SPR-40k) was detected under conditions which reduced SPR photolabeled with [¹²⁵I]BPA³-SP to peptides of less than 10 kDa. Very high concentrations of V8 produced, in addition to the major band at 40 kDa, minor bands at about 3 and 10 kDa. Endo F digestion of BPA³-SPR-40k produced a 3.5-kDa fragment, BPA³-SPR-3.5k. The same fragment was produced by double digestion with Endo F and V8 protease of SPR photolabeled with [¹²⁵I]BPA³-SP.

V8 protease-digested fragments of [¹²⁵I]BPA³-SP-labeled SPR were separated by HPLC. A major peak containing >70% of eluted radioactivity passed through without being retarded by the reverse-phase column. These fractions were dried, digested with Endo F, and reanalyzed by HPLC. The Endo F-treated sample (BPA³-SPR-3.5k) was retained by the HPLC column and eluted at 30% solvent B (Fig. 7). BPA³-SPR-3.5k and BPA³-SP were separable by HPLC. A control sample (identically treated in the absence of Endo F) still passed through the HPLC column without retention.

Trypsin Digestion of the Labeled SP Receptor—Double digestion of [¹²⁵I]BPA³-SP-labeled SPR with Endo F and trypsin revealed the same pattern of radioactive fragments as seen with trypsin digestion alone. However, double digestion (with Endo F and trypsin) of [¹²⁵I]BPA³-SP-labeled SPR produced a different pattern of fragments than that seen with trypsin treatment alone. As seen with Endo F and V8, digestion of the major high molecular mass (>45 kDa) tryptic fragment of [¹²⁵I]BPA³-SP-labeled SPR with Endo F converted it to a much smaller fragment.

DISCUSSION

Replacement of amino acid residues at the third (Lys³) or eighth (Phe⁸) positions of SP by BPA and addition of Tyr at the

NH₂-terminal (Tyr⁰) gave analogs (Fig. 1) which triggered calcium responses of P388D₁ cells (Fig. 2) with the same potency as the parent peptide. The calcium response was inhibited by CP-96345, a specific SPR antagonist. Binding of [¹²⁵I]-labeled BPA³-SP and BPA³-SP to P388D₁ cells was blocked by cold SP and CP-96345 at nM concentrations (Table I). The two photolabile ligands thus are high affinity full agonists of the SPR of P388D₁ cells. Previous studies showed that Phe⁸ of SP could be structurally modified without a marked decrease in activity on affinity on several bioassays (Lee *et al.*, 1983; Maggio, 1988; Viger *et al.*, 1983). Boyd *et al.* (1991a, 1991b) have demonstrated that replacement of Phe⁸ of SP by BPA is well tolerated in binding affinity and biological activity. The results of the present study illustrate that substitution of Lys³ of SP with BPA also maintains biological activity and binding affinity at the SPR of murine P388D₁ cells. To facilitate radioiodination, we added a tyrosine at the NH₂ terminus of the peptide; the Tyr⁰ peptides also retain full biological activity and binding affinity (Sachais *et al.*, 1993; Cascieri *et al.*, 1994).

Upon near UV irradiation (>310 nm), [¹²⁵I]-labeled BPA³-SP and BPA³-SP are photoincorporated into a major broad radio-labeled band of ~75 kDa in P388D₁ cells (Fig. 3). The broad range of molecular mass reflects heterogeneous glycosylation, as Endo F treatment dramatically converted the broad 75 kDa band to a sharp one of ~42 kDa (Fig. 4). This size is consistent with the value deduced from the cDNA sequence for the mouse SPR (Sundelin *et al.*, 1992). The sensitivity of photolabeling with these ligands to SP and CP-96345 (Table I, Fig. 3) further indicated that BPA³-SP and BPA³-SP were cross-linked with the SPR.

This intact cell-photolabeling technique demonstrates that the SPR expressed in this natural (*i.e.* nontransfected) cell line is highly glycosylated. Reports of photolabeling of SPR prepared from various other sources suggests a heterogeneity of molecular size. Dam *et al.* (1987), using a photoreactive SP analog in which Phe⁸ was replaced by *p*-azidophenylalanine, demonstrated specific photolabeling of a single polypeptide, ~46 kDa, in a rat brain membrane preparation. Boyd *et al.* (1994) reported that the molecular mass of SPR in rat tissues labeled with [¹²⁵I]-3-(3-iodophenyl-4-hydroxyphenyl)propionyl-Lys³-BPA³-SP varied from 53 and 46 kDa for submaxillary or parotid gland to 72 kDa for large intestine and 90 kDa for striatum or olfactory bulb. Deglycosylation of each of these photolabeled receptors from different tissues yielded a discrete radiolabeled band of ~46 kDa, while in salivary gland an additional band at ~36 kDa was also observed.

Limited V8 digestion of [¹²⁵I]BPA³-SP-labeled SPR implies that the smallest labeled complex, BPA³-SPR-3.2k, represents the interaction site of the ligand and receptor, as all other fragments are converted to the 3.2-kDa fragment at high concentrations of protease. Since BPA³-SP has a molecular mass of ~1.7 kDa, a SPR V8 fragment peptide with molecular mass of ~1.5 kDa is involved in the BPA³-SPR-3.2k complex. DTNB reaction indicates that this peptide contains a cysteine residue. The V8 digestion map of the mouse SPR deduced from its cDNA sequence shows only four cysteine-containing peptides, with values of 10.7 kDa (SPR 79–172, 2 Cys), 1.3 kDa (SPR 173–183, 1 Cys), 3.8 kDa (SPR 194–227, 1 Cys), and 11.1 kDa (SPR 239–312, 6 Cys). The two larger fragments (*M_r* > 10 kDa) are excluded based on their molecular masses. Of the remaining two, the smaller (1.3 kDa) is clearly a much better candidate than the larger (3.8 kDa) for the ~1.5-kDa fragment deduced from the V8 digestion studies.

Consistent with the size prediction, HPLC elution behavior confirms that SPR 173–183 (1.3 kDa) is the photolabeled receptor fragment. SPR 173–183 is a part of the relatively polar second

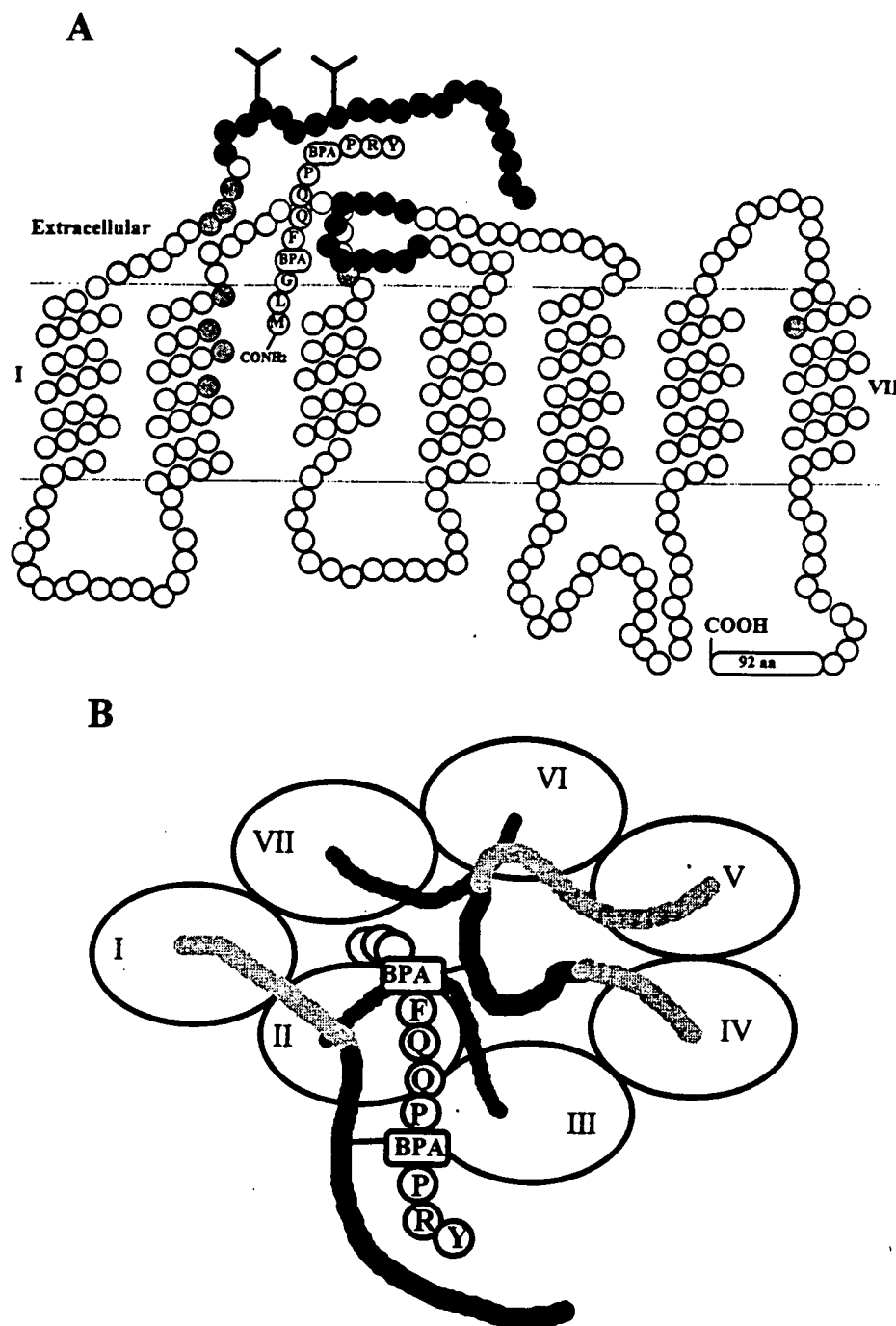


FIG. 8. Schematic model of the peptide agonist binding site of the murine SP receptor. Black circles represent the contact regions of SPR with SP analogs (BPA³ and BPA⁸). The third and the eighth positions of SP, respectively, interact with the NH₂-terminal extracellular tail (SPR 1–21, MDNVLPVDSDF-PNTSTNTSE) and second extracellular loop (SPR 173–183, TMPSRVVCMIE) of the SP receptor. Shaded circles indicate residues essential for high affinity binding of SP as identified by site-directed mutagenesis. A, view in the plane of the bilayer; B, view from the extracellular side, normal to the plane of the bilayer. See text for further explanation.

extracellular domain of the SPR (between transmembrane domain (TM) IV and TMV), with a hydrophobic index (Engelman *et al.*, 1986) of -1.8 . The addition of such a fragment to BPA⁸-SP (hydrophobic index -15.1) would have little effect on HPLC elution position, as is observed experimentally (Fig. 6). Both the tracer alone, and its photoadduct with the receptor fragment, elute as sharp peaks at the same ($\sim 33\%$ solvent B) position in the solvent gradient. In sharp contrast, SPR 194–227 (~ 3.8 kDa) is comprised mainly of the very hydrophobic TMV region of the receptor and has a hydrophobic index of 35. Hydrophobic peptides elute from reverse-phase HPLC columns as relatively broad peaks at very high solvent concentrations. For example, a photolabeled transmembrane domain (α -M4, residues 401–428, hydrophobic index = 36.9) from the *Torpedo* nicotinic acetylcholine receptor, with very similar molecular weight and amino acid composition to SPR 194–227, elutes in a very broad peak at about

74% solvent B in the same HPLC system (Blanton and Cohen, 1994). Such behavior is inconsistent with the reverse-phase-HPLC elution of BPA⁸-SPR-3.2k (Fig. 6). Thus, molecular weight, HPLC elution behavior, and presence of cysteine, taken together, establish that the SPR region photolabeled by BPA⁸-SP is SPR 173–183 of the second extracellular loop, whose primary sequence is TMPSRVVCMIE. Using a different SP tracer containing BPA at position 8, Boyd *et al.* (1993) also found labeling of the second extracellular loop of rat SPR in transfected hamster cells, a finding consistent with the present results. Because the radiolabel in these probes is located at a site distinct from the photoreactive amino acid, radiochemical sequencing cannot be used to define the specific amino acid of the SPR labeled by BPA.

Limited V8 digestion of [¹²⁵I]BPA³-SP-labeled SPR suggested that the glycosylated peptide complex, BPA³-SPR-40k, represents the interaction site of the ligand and receptor be-

cause Endo F plus V8 mixed digestion shifted the 40-kDa complex to a much smaller fragment, BPA³-SPR-3.5k. This is further confirmed by HPLC analysis. BPA³-SPR-40k passed through the reverse-phase column in the void volume, behavior common to very polar biopolymers such as carbohydrates. Deglycosylation of BPA³-SPR-40k converted the complex to a smaller peptide (~3.5 kDa) which was retained by the reverse-phase column and eluted by the solvent gradient at about 30% solvent B. There are two potential sites (N-X-S/T) for N-linked glycosylation in the SP receptor. Both are located in the NH₂-terminal extracellular tail of the receptor, based both on primary sequence (Sundelin *et al.*, 1992) and experimental results (Boyd *et al.*, 1991b, 1994). The V8 digestion map of the murine SPR indicated that NH₂-terminal peptide (SPR 1–21, ~2.3 kDa) contains two N-linked glycosylation sites, while all other fragments have none. The hydrophobicity index of this (deglycosylated) peptide is -27.3, which predicts that the deglycosylated receptor fragment cross-linked with BPA³-SP would elute earlier than the free photoprobe. Consistent with this prediction, the complex does elute earlier in the solvent gradient than [¹²⁵I]BPA³-SP. Thus, molecular size of the complex, the presence of carbohydrate, and HPLC elution behavior, taken together, establish that the NH₂-terminal extracellular tail of the receptor (SPR 1–21, whose primary sequence is MDNVLPVDS-DLFPNTSTNTSE) is the insertion site of [¹²⁵I]BPA³-SP.

Photoaffinity labeling identifies receptor domains in close proximity to the bound photoligand. Another approach to receptor-ligand interactions, site-directed mutagenesis, identifies domains necessary for function, but which are not necessarily proximal to the site of that function. Chimeras of the substance P receptor with other tachykinin receptors (*e.g.* substance K receptor) demonstrated the agonist ligand specificity of the tachykinin receptors is mainly determined by the region around TMII TMIV and also partly by the extracellular NH₂-terminal domain of the receptors (Yoshifumi *et al.*, 1992). Fong *et al.* (1992b) found that extracellular domains of SPR (also known as NK-1R), including a segment of NH₂-terminal tail and the first extracellular loop were essential for high affinity binding of agonist peptides. Furthermore, they identified several residues in the NH₂-terminal domain (Asn²³, Gln²⁴, and Phe²⁵), first extracellular (also known as E2) loop (Asn⁹⁶, His¹⁰⁸), and part of second extracellular (also known as E3) loop (Ser¹⁷⁶-Glu¹⁸³) which are required for high affinity binding of peptides (Fong *et al.*, 1992a). Other mutagenesis studies demonstrated that residues in TMII (Asn⁸⁵, Asn⁸⁹, Tyr⁹²) and TMVII (Tyr²⁸⁷) are also required for high affinity binding of peptide agonists (Huang *et al.*, 1994b). Analysis of SP analogs further suggested the COOH-terminal carboxamide of SP may interact with Asn⁸⁶ in the second transmembrane domain (Huang *et al.*, 1994b). Taken together, these data demonstrate that both the extracellular and transmembrane domains of SPR are important for the peptide binding. The present studies identify the interaction sites of the third (BPA³) and eighth (BPA⁸) positions of SP as (*i.e.* BPA³ and BPA⁸ contact and photolabel) the NH₂-terminal extracellular tail (SPR 1–21) and the second extracellular loop (SPR 173–183) of the receptor, respectively. The results of the present photolabeling experiments and those of previous mutagenesis experiments are distinct, in that the different regions of the SPR are identified, but not inconsistent. The photolabeling results do not match the predictions of a graphics-computer-generated model (Trumpf-Kallmeyer *et al.*, 1994) of SP bound to its receptor.

Combining the present results with those of previous mutagenesis studies, a model of the agonist peptide-binding site of the SPR can be constructed (Fig. 8). In this model, the COOH-terminal hydrophobic sequence -GLM-NH₂ of SP inserts into a

hydrophobic ligand binding pocket between the transmembrane domains and between the extracellular surface and center of the bilayer. This binding pocket is formed by TMII and TMVII with contributions from other transmembrane domains. The carboxamide penetrates to the level of and interacts with Asn⁸⁵ (Huang *et al.*, 1994b). Other than this COOH-terminal tail, the remainder of the SP molecule interacts with amino acids on the extracellular face on the receptor. Specifically, position 8 of SP interacts with the second extracellular loop (SPR 173–183) and position 3 of SP with the NH₂-terminal extracellular tail (SPR 1–21). These regions of the SPR are highly conserved across species; 10 of 11 amino acids of SPR 173–183 and 18 of 21 amino acids of SPR 1–21 are invariant across the four mammalian species whose SPR cDNA sequences have been reported (Gerard *et al.*, 1993). The binding site for specific, high affinity nonpeptide antagonists of the SPR is at a distinct location (Cascieri *et al.*, 1994; Fong *et al.*, 1992a, 1992b, 1993, 1994a, 1994b; Gether *et al.*, 1993a, 1993b, 1993c, 1994; Huang *et al.*, 1994a; Jensen *et al.*, 1994; Sachais *et al.*, 1993; Yokota *et al.*, 1992; Zoffmann *et al.*, 1993).

Studies of other G-protein-coupled receptors have demonstrated that those which bind larger (>10 kDa) agonists have agonist-binding sites within their NH₂-terminal extracellular domains. In contrast, receptors of this superfamily which bind smaller (<0.5 kDa) nonpeptide agonists have agonist-binding sites deep within the bilayer between the transmembrane domains (Bockaert, 1991; Dohlman *et al.*, 1991). The smallest neuropeptide, thyrotropin-releasing hormone (360 Da) apparently also binds within this same region (Perlman *et al.*, 1994). Recently Gerszten *et al.* (1994) found that the specificity of thrombin receptors for peptide agonists was determined by the extracellular face of the receptor. Substance P, a peptide agonist of intermediate size, apparently interacts with both the extracellular region and transmembrane region of its receptor. Thus, the regions of interaction between SP and its receptor include elements of both the large and small agonist-receptor systems. Other bioactive peptides among the dozens in this intermediate size range may similarly interact with both the extracellular and transmembrane domains of their own G-protein-coupled receptors.

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REFERENCES

- Baldwin, J. M. (1994) *Curr. Opin. Cell Biol.* **6**, 180–190
- Blanton, M. P., and Cohen, J. B. (1994) *Biochemistry* **33**, 2859–2872
- Bockaert, J. (1991) *Curr. Opin. Neurosci.* **1**, 32–42
- Boyd, N. D., Macdonald, S. G., Kage, R., Lubner-Narod, J., and Leeman, S. E. (1991a) *Ann. New York Acad. Sci.* **632**, 79–93
- Boyd, N. D., White, C. F., Cerpa, R., Kaiser, E. T., and Leeman, S. E. (1991b) *Biochemistry* **30**, 336–342
- Boyd, N. D., Kage, R., Dumas, J. J., Krause, J. E., and Leeman, S. E. (1993) *J. Neurochem. Suppl.* **61**, S63
- Boyd, N. D., Kage, R. K., and Leeman, S. E. (1994) *The Tachykinin Receptors* (Buck, S. H., ed) pp. 219–236, Humana Press, Totowa, NJ
- Cascieri, M. A., Macleod A. M., Underwood, D., Shiao, L. L., Ber, E., Sadowski, S., Yu, H., Merchant, K. J., Swain, C. J., Strader, C. D., and Fong, T. M. (1994) *J. Biol. Chem.* **269**, 6587–6591
- Dam, T.-V., Escher, E., and Quirion, R. (1987) *Biochem. Biophys. Res. Commun.* **149**, 297–303
- Dawe, C. J., and Potter, M. (1957) *Am. J. Pathol.* **33**, 603
- Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) *Annu. Rev. Biochem.* **60**, 653–688
- Dorman, G., and Prestwich, G. D. (1994) *Biochemistry* **33**, 5661–5673
- Engelman, D. M., Steitz, T. A., and Goldman, A. (1986) *Annu. Rev. Biophys. Biophys. Chem.* **15**, 321–353
- Fong, T. M., Huang, R. R. C., and Strader, C. D. (1992a) *J. Biol. Chem.* **267**, 25664–25672
- Fong, T. M., Yu, H., Huang, R. R. C., and Strader, C. D. (1992b) *Biochemistry* **31**, 11806–11811
- Fong, T. M., Cascieri, M. A., Yu, H., Bansal, A., Swain, C., and Strader, C. D. (1993) *Nature* **362**, 350–353
- Fong, T. M., Yu, H., Cascieri, M. A., Underwood, D., Swain, C. J., and Strader, C. D. (1994a) *J. Biol. Chem.* **269**, 2728–2732

- Fong, T. M., Yu, H., Cascieri, M. A., Underwood, D., Swain, C. J., and Strader, C. D. (1994b) *J. Biol. Chem.* **269**, 14957-14961
- Fournier, A., Couture, R., Regoli, D., Gendreau, M., and St-Pierre, S. (1982) *J. Med. Chem.* **25**, 64-68
- Gerard, N. P., Bao, L., He, X. P., and Gerard, C. (1993) *Regul. Peptides* **43**, 21-35
- Gerszten, R. E., Chen, J., Ishii, M., Ishii, K., Wang, L., Nanavicz, T., Turck, C. W., Vu, T.-K. H., and Coughlin, S. R. (1994) *Nature* **368**, 648-651
- Gether, U., Johansen, T. E., and Schwartz, T. W. (1993a) *J. Biol. Chem.* **268**, 7893-7898
- Gether, U., Johansen, T. E., Snider, R. M., Lowe, J. A., III, Nakanishi, S., and Schwartz, T. W. (1993b) *Nature* **362**, 345-347
- Gether, U., Yokota, Y., Edmonds-Alt, X., Brelviere, J. C., Lowe, J. A., III, Snider, R. M., Nakanishi, S., and Schwartz, T. W. (1993c) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6194-6198
- Gether, U., Edmonds-Alt, X., Brelviere, J. C., Fujii, T., Hagiwara, D., Pradier, L., Garret, C., Johansen, T. E., and Schwartz, T. W. (1994) *Mol. Pharmacol.* **45**, 500-508
- Huang, R. R. C., Yu, H., Strader, C. D., and Fong, T. M. (1994a) *Mol. Pharmacol.* **45**, 690-695
- Huang, R. R. C., Yu, H., Strader, C. D., and Fong, T. M. (1994b) *Biochemistry* **33**, 3007-3013
- Jensen, C. J., Gerard, N. P., Schwartz, T. W., and Gether, U. (1994) *Mol. Pharmacol.* **45**, 294-299
- Kage, R. K., Leeman, S. E., and Boyd, N. D. (1993) *J. Neurochem.* **60**, 347-351
- Kauer, J. C., Erickson-Viitanen, S., Wolfe, H. R., Jr., and DeGrado, W. F. (1986) *J. Biol. Chem.* **261**, 10695-10700
- Laemmli, U. K. (1970) *Nature* **227**, 680-685
- Lee, C.-M., Javitch, J. A., and Snyder, S. H. (1983) *Mol. Pharmacol.* **23**, 563-569
- Li, Y.-M., Wingrove, D. H., Too, H.-P., Marnierakis, M., Stimson, E. R., Strichartz, G. R., and Maggio, J. E. (1995) *Anesthesiology*, in press
- Maggio, J. E. (1988) *Annu. Rev. Neurosci.* **11**, 13-28
- Maggio, J. E., Stimson, E. R., Ghilardi, J. R., Allen, C. J., Dahl, C. E., Whitcomb, D. C., Vigna, S. R., Vinters, H. R., Labenaki, M. E., and Mantyh, P. W. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 5462-5466
- Marley, P. (1984) *Carlsberg Res. Commun.* **49**, 591-596
- Otsuka, M., and Yoshioka, K. (1993) *Physiol. Rev.* **73**, 229-308
- Perlman, J. H., Thaw, C. N., Laakkonen, L., Bowers, C. Y., Osman, R., and Gershengorn, M. C. (1994) *J. Biol. Chem.* **269**, 1610-1613
- Pernow, B. (1983) *Pharmacol. Rev.* **35**, 85-141
- Persico, F. J., Kimball, E. S., and Vaught, J. L. (1988) *J. Immunol.* **141**, 3564-3569
- Sachais, B. S., Snider, R. M., Lowe, J. A., III, and Krause, J. E. (1993) *J. Biol. Chem.* **268**, 2319-2323
- Shoelson, S. E., Lee, J., Lynch, C. S., Backer, J. M., and Pilch, P. F. (1993) *J. Biol. Chem.* **268**, 4085-4091
- Sundelin, J. B., Provvedini, D. M., Wahlestedt, C. R., Laurell, H., Pohl, J. S., and Peterson, P. A. (1992) *Eur. J. Biochem.* **203**, 625-631
- Too, H.-P., and Maggio, J. E. (1991) *Methods. Neurosci.* **6**, 232-247
- Trumpp-Kallmeyer, S., Hoffack, J., and Hibert, M. (1994) *The Tachykinin Receptors* (Buck, S. H., ed) pp. 237-255, Humana Press, Totowa, NJ
- Viger, A., Beaujouan, J. C., Torrens, Y., and Glowinski, J. (1983) *J. Neurochem.* **40**, 1030-1038
- Yokota, Y., Akazawa, C., Ohkubo, H., and Nakanishi, S. (1992) *EMBO J.* **11**, 3585-3591
- Zoffmann, S., Gether, U., and Schwartz, T. W. (1993) *FEBS Lett.* **336**, 506-510

Human Parathyroid Hormone: Amino-Acid Sequence of the Amino-Terminal Residues 1-34

(automated Edman degradation/mass spectrometry/calcium metabolism/metabolic bone disease)

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ABSTRACT Human parathyroid hormone has been isolated in highly purified form from human parathyroid adenomas. The primary sequence of the amino-terminal residues of the human hormone was obtained by automated degradation with a Beckman Sequencer. The phenylthiohydantoin amino acids were identified by gas chromatography and mass spectrometry. The first 34 residues of human parathyroid hormone differ from the bovine hormone by six residues, and from the porcine hormone by five residues. The amino-terminal residue is aspartic acid, similar to the porcine parathyroid hormone; the bovine parathyroid hormone contains an amino-terminal asparagine. Human parathyroid hormone contains two methionine residues, similar to the bovine species, whereas porcine parathyroid hormone contains a single methionine residue. Amino-acid residues in the first 34 that are unique to the human sequence include an asparagine at position 1, glutamine at position 22, lysine at position 28, and a leucine at position 30.

During the last few years, a significant core of information has been obtained by several laboratories on the chemistry, biosynthesis, and secretion of parathyroid hormone (PTH). These studies have indicated that this hormone is initially synthesized as a prohormone, proparathyroid hormone (1-4). Proparathyroid hormone contains about 106 amino acids, and has an apparent molecular weight of 12,500 (4). The prohormone is rapidly converted into the storage or glandular form of the hormone, which consists of 84 amino acids and has a molecular weight of 9500. The complete amino-acid sequences of the 84 amino-acid parathyroid hormone from bovine (5, 6) and porcine (7) species have been reported. After appropriate physiological stimuli, the 9500 molecular weight form of the parathyroid hormone is secreted into the circulation (8). Shortly after entering the peripheral circulation, the glandular form of the hormone is cleaved into smaller fragments. Gel filtration of human hyperparathyroid serum by several investigators has revealed a major immunoreactive fragment(s), with a molecular weight of 5000-8000, and several minor components (8-10). Immunochemical heterogeneity of the circulating human parathyroid hormone, presumably due to the different molecular forms of PTH, was initially reported by Berson and Yalow (11), and has been confirmed by others (12, 13). The specific site(s) of cleavage in the 84-amino-acid polypeptide chain of the parathyroid hormone in the general circulation is unknown. A biologi-

cally active peptide fragment of bovine PTH, prepared by dilute acid cleavage, has been reported (14, 15), a result indicating that the intact 84-amino-acid polypeptide is not needed for biological activity. This peptide has been identified as the amino-terminal peptide of the hormone, and is composed of the initial 30 residues of the sequence (15). Synthetic peptides of the first 34 residues of the bovine hormone (16) and the initial 30 residues of the porcine hormone (17) have been prepared and are biologically active, thereby confirming the localization of the biologically active region of the parathyroid hormone to the amino-terminal third of the 84-amino-acid polypeptide chain.

The purpose of this communication is to report the amino-terminal sequence of the first 34 residues of human parathyroid hormone, and to compare the amino-terminal sequence of the human hormone to that of the bovine and porcine species.

MATERIALS AND METHODS

The human parathyroid hormone used in these studies was isolated from parathyroid adenomas obtained from patients undergoing surgery for hyperparathyroidism. Dried, defatted parathyroid tissue was initially extracted with 8 M urea in 0.2 N hydrochloric acid, and fractionated with ether, acetic acid, sodium chloride, and trichloroacetic acid (TCA powder) (18). The TCA powder was further purified by gel filtration, followed by ion-exchange chromatography on CM-sephadex with an ammonium acetate gradient. Isolation of the hormone was monitored by radioimmunoassay and disc-gel electrophoresis. The procedures used in the isolation and characterization of the hormone will be described in detail in a separate report.

Amino-acid analyses were performed on a Beckman-Spinco automatic amino-acid analyzer, model 120B or 121 adapted for high sensitivity or with a Durrum model 500 analyzer. Analytical disc-gel electrophoresis was performed in 8 M urea at pH 4.4 (19). Immunoassays were performed by the procedure of Arnaud *et al.* (20).

Automated Edman degradations were performed with the Beckman Sequencer, model 890B, in 1 M Quadrol buffer. The phenylthiohydantoin (PTH) amino acids were identified by regeneration to the constituent amino acid by hydrolysis with hydroiodic acid for 20 hr at 130° (21), gas-liquid

Abbreviation: PTH, parathyroid hormone



FIG. 1. Disc-gel electrophoresis of purified human (left) and bovine (right) parathyroid hormones.

chromatography (22, 23), and mass spectrometry (24–26). Chemical ionization mass spectrometry was performed on a Finnigan mass spectrometer equipped with a PDP-8/e Digital computer, and a Complot Plotter. Isobutane was used as the carrier gas, and the source was maintained at 200°. The samples were applied by a direct insertion probe, and the probe was heated from 30° to 250° over a 90-sec period. Electron impact mass spectrometry was performed on an LKB mass spectrometer, model 9000, with a direct insertion probe and an electron energy of 70 eV.

RESULTS

The purified human parathyroid hormone migrated as a single component on disc-gel electrophoresis, with a mobility identical to that of the bovine parathyroid hormone (Fig. 1). Amino-terminal analysis of the purified peptide by the Edman technique revealed serine.

350 nanomoles of the purified hormone were degraded on the Beckman Sequencer by use of a single cleavage of hepto-

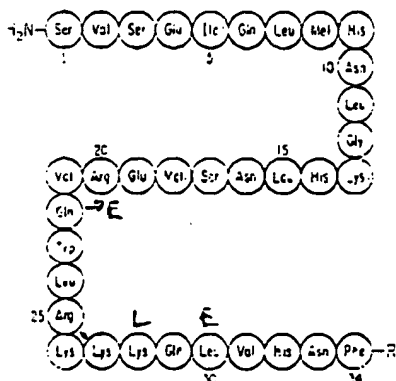


FIG. 2. Amino-acid sequence of the amino-terminal 34 residues of human parathyroid hormone.

fluorobutyric acid at each degradation. The results of the degradation of the first 34 residues of the human parathyroid hormone are shown in Fig. 2. The chemical ionization mass spectra of the phenylthiohydantoin (PTH) amino acids obtained at each of the 34 steps in the sequence are shown in Fig. 3. A "quasimolecular" (QM^+) or major fragmentary ion is observed in each spectrum (25). At step 12 in the sequence, a quasimolecular ion for glycine (m/e 192) and leucine (m/e 249) are observed (Fig. 3). Quantitation by the gas chromatography method of glycine (0.28 μ M) and leucine (0.09 μ M) permits definitive identification of glycine as the twelfth amino acid in the sequence, with the leucine resulting from overlap from step 11 (Fig. 2). The ion at m/e 292 and 293 in the mass spectra of step 20 are contaminant ions often observed in variable amounts in the aqueous layer of the Edman reaction. Leucine/isoleucine and lysine/glutamine yield identical masses of m/e 249 and m/e 264, respectively, on chemical ionization mass spectrometry. Lysine, however, can be distinguished from glutamine by the fragmentary ion at m/e 306, as illustrated in the spectra of residues 26, 27, and 28. Lysine/glutamine and leucine/isoleucine were also readily differentiated by gas chromatography on the CFC blend (23) and by electron impact mass spectrometry (24, 25).

These combined results provided a single unique sequence for the first 34 residues of human parathyroid hormone (Fig. 2).

DISCUSSION

The amino-acid sequence of the first 34 residues of human parathyroid hormone is of major importance, since previous studies of the bovine and porcine species have indicated that this is the biologically active region of the native hormone. The first 34 residues of human PTH differ from bovine PTH by six residues, and porcine PTH by five residues (Fig. 4). The amino-terminal 15 residues of human and porcine PTH are identical; however, bovine PTH differs from human and porcine PTH in position 1 and 7, where alanine substitutes for serine and leucine replaces phenylalanine (Fig. 4). In the remaining 16–34 region, human PTH differs from porcine PTH by five residues, and from bovine PTH by four residues (Fig. 4). Human PTH contains two methionine residues—similar to the bovine species—whereas porcine PTH contains a single methionine at position 8 (Fig. 4). The human sequence is unusual in that it contains four consecutive basic residues (arginine residue 25, and lysine residues 26–29). Amino-acid residues in the first 34 that are unique to the human sequence include an asparagine at position 16, glutamine at position 22, lysine at position 28, and a leucine at position 30.

One of the major problems in the clinical assessment of patients with disorders of mineral metabolism has been the difficulties encountered with the radioimmunoassay of human parathyroid hormone. There have been two basic problems with the immunoassay of PTH. The first problem, as discussed above, has been the presence in the peripheral circulation of peptide fragments of the 84 amino-acid polypeptide chain (8–10). Antisera from various laboratories undoubtedly have immunological determinants for different regions of the intact molecule, thus leading to variable and sometimes inconsistent results when applied to the measurement of PTH circulating in human blood (27). In addition, the differentiation by immunoassay of biologically active

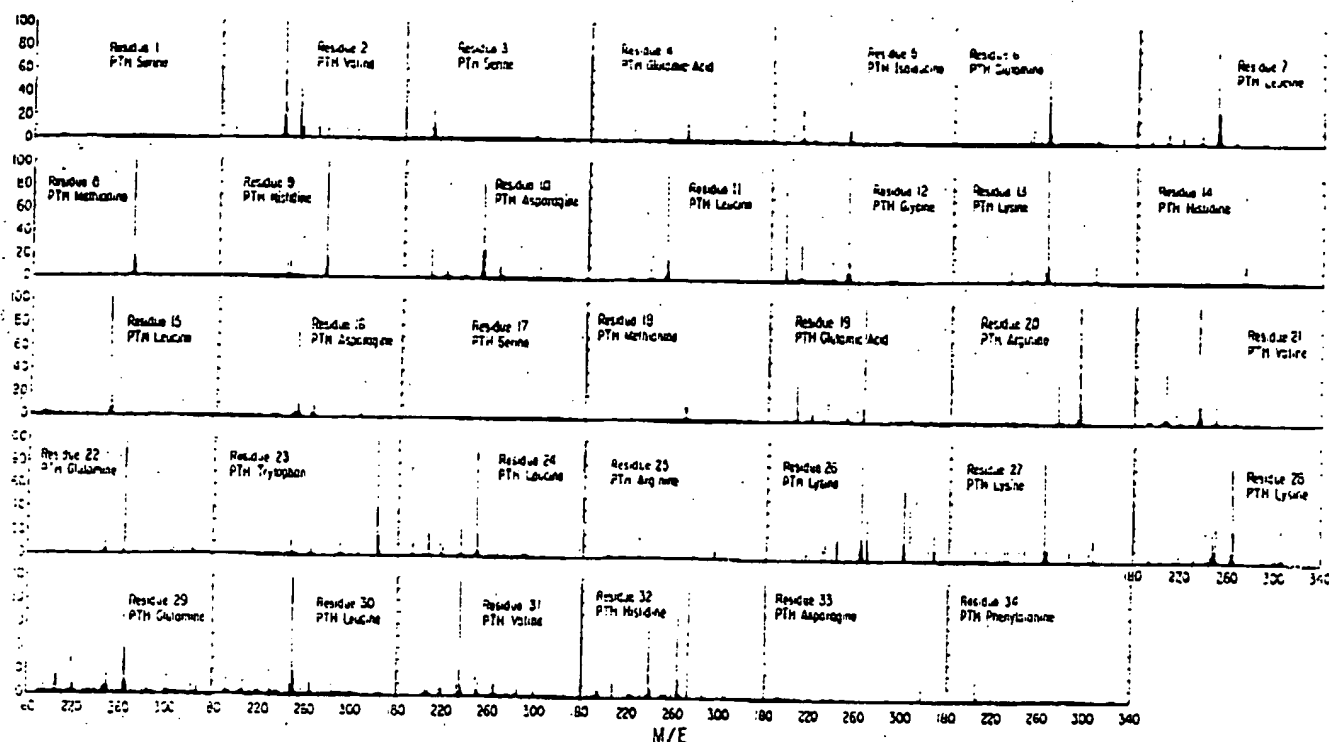


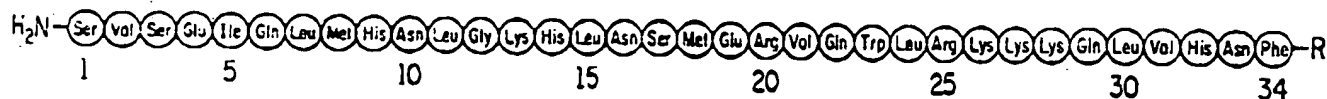
FIG. 3. Mass spectra of the phenylthiohydantoin (PTH) amino acids obtained during the automated Edman degradation of the amino-terminal 34 residues of the human parathyroid hormone.

amino-terminal fragments from inactive fragments has so far been impossible. The second difficulty has been the utilization of heterologous assays that use radioactively labeled bovine hormone as the tracer, and antibodies prepared against the bovine or porcine hormone (28-30, 20). The sensitivities of these assays are variable, and depend on the cross reactivity of the particular antiserum with the human hormone. As noted above, the human sequence in only the initial third of the molecule differs from the bovine by six residues and the porcine by five amino acids.

Habener *et al.* (31) have attempted to circumvent some of these problems with the immunoassay by the development of

amino- and carboxyl-specific antisera. These investigators have used an antibody prepared against the bovine hormone, and have absorbed their antiserum with either the synthetic 1-34 bovine fragment, or a 53-84 fragment prepared by chemical cleavage of the native bovine hormone. The amino-terminal specific antiserum was further characterized by displacement with synthetic bovine fragments, and the recognition site of this absorbed antiserum was shown to be directed toward residues 14-19 in the bovine sequence. Using this approach, they have concluded that the major fragment in the human circulation is carboxyl-terminal, and biologically inactive. They were, however, unable to identify the amino-

HUMAN PARATHYROID HORMONE



BOVINE PARATHYROID HORMONE



PORCINE PARATHYROID HORMONE



FIG. 4. Comparison of the amino-acid sequence of the amino-terminal 34 residues of human-, bovine-, and porcine-parathyroid hormones.

terminal fragment in the circulation of human subjects. This may be due either to rapid clearance of the amino-terminal fragment from the circulation, or to poor cross-reactivity of the amino-terminal specific bovine antiserum with the amino-terminal region of the human hormone. It is of interest that the human sequence differs in the 14-19 region from the bovine hormone by the substitution at residue 16 of an asparagine for a serine residue (Fig. 4). The significance of this substitution in the human hormone to the results obtained by Habener *et al.* with their amino-terminal specific bovine antiserum is unknown. Canterbury and Reiss have reported results on the nature of the circulating fragment of the parathyroid hormone that are in contrast to those reported by Habener *et al.* Using an antiserum prepared against bovine parathyroid hormone, these investigators have identified three different immunochemical forms of the parathyroid hormone in the peripheral circulation of hyperparathyroid patients (32). The molecular weights of these three components, as determined by gel filtration, were 9500 (presumably glandular PTH), 7000-7500, and 4500-5000. Recently, these investigators have directly assessed the biological activity of these three fragments in a renal adenylate cyclase system (33). Both the 9500 and the 4500-5000 fragment stimulated the adenylate cyclase system, whereas the 7000-7500 component was inactive. These results are consistent with the presence of an amino-terminal biologically active fragment of PTH of about one-half the size of the glandular hormone in human hyperparathyroid serum.

The determination of the amino-terminal sequence of the human parathyroid hormone will now permit the synthesis of peptides based on the human sequence for both clinical and investigative use. Synthetic fragments, as well as chemical analogues, will permit more definitive studies to be performed on the chemistry of the human hormone, including the specific residues and the minimum length of the polypeptide chain that is required for biological activity. In addition, these synthetic fragments will enable investigators to characterize the heterologous antisera currently in use in the immunoassay, and to develop specific antisera directed toward the amino-terminal region of the human hormone. Antisera based on the human sequence will enable more detailed studies to be performed on the nature of the circulating hormone in man, and its role in calcium homeostasis and metabolic bone disease.

An international cooperative effort has made the work reported in this manuscript possible. More than 150 individual laboratories, physicians, surgeons, and pathologists donated human parathyroid tissue for use in the extraction and purification of the human parathyroid hormone that was used in the determination of the amino-terminal sequence of the human hormone. Space does not permit a listing of their names here; however, they are represented by human PTH study groups from Australia, Canada, Europe (Belgium, Germany, Holland, and Switzerland), France, Japan, Mexico, Spain, Sweden, and the United States. A great deal of the credit for the results reported in this manuscript is due to the untiring efforts of these individuals. We also thank Drs. Henry Falas and Bill Milne for their assistance in the mass spectrometric analyses. The excellent technical assistance of Mr. Wayne Blanchard, Mrs. M. Juliariva, Mrs. Judy Larsen, Miss Ann Kelly, and Miss Janice Leffler is gratefully acknowledged. This work was supported in part by grants from the U.S. Public Health Service (NIH-Am 12302) and from the Mayo Foundation.

1. Hamilton, J. W., Spierio, F. W., MacGregor, R. R. & Cohn, D. V. (1971) *J. Biol. Chem.* 246, 3224-3233.
2. Hamilton, J. W., MacGregor, R. R., Chu, L. L. H. & Cohn, D. V. (1971) *Endocrinology* 89, 1440-1447.

3. Kemper, B., Habener, J. F., Potts, J. T. & Rich, A. (1972) *Proc. Nat. Acad. Sci. USA* 69, 643-647.
4. Cohn, D. V., MacGregor, R. R., Chu, L. L. H., Kimmel, J. R. & Hamilton, J. W. (1972) *Proc. Nat. Acad. Sci. USA* 69, 1521-1525.
5. Brewer, H. B., Jr. & Ronan, R. (1970) *Proc. Nat. Acad. Sci. USA* 67, 1862-1869.
6. Niall, H. D., Keutmann, H., Sauer, R., Hogan, M., Dawson, B., Aurbach, G. & Potts, J. T., Jr. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* 351, 1586-1588.
7. O'Riordan, J. L. H., Woodhead, J. S., Robinson, C. J., Parsons, J. A., Keutmann, H., Niall, H. & Potts, J. T. (1971) *Proc. Roy. Soc. Med.* 64, 1263-1265.
8. Habener, J. F., Powell, D., Murray, T. M., Mayer, G. P. & Potts, J. T., Jr. (1971) *Proc. Nat. Acad. Sci. USA* 68, 2986-2991.
9. Canterbury, J. M. & Reiss, E. (1971) *J. Lab. Clin. Med.* 73, 814.
10. Arnaud, C. D., Goldsmith, R. G., Sizemore, G. W., Bordier, P., Oldham, S. B., Bishoff, J. & Larsen, J. A. (1973) "Clinical Aspects of Metabolic Bone Disease," *Excerpta Med.*, in press.
11. Berson, S. A. & Yalow, R. S. (1968) *J. Clin. Endocrinol. Metab.* 28, 1037-1047.
12. Arnaud, C. D., Tsao, H. S. & Oldham, S. B. (1970) *Proc. Nat. Acad. Sci. USA* 67, 415-422.
13. Sherwood, L. M., Rodman, J. S. & Lundberg, W. B. (1970) *Proc. Nat. Acad. Sci. USA* 67, 1631-1638.
14. Rasmussen, H. & Craig, L. C. (1962) *Recent Progr. Horm. Res.* 18, 269-289.
15. Keutmann, H. T., Dawson, B. F., Aurbach, G. D. & Potts, J. T., Jr. (1972) *Biochemistry* 11, 1973-1979.
16. Potts, J. T., Jr., Tregar, G. W., Keutmann, H. T., Niall, H. D., Sauer, R., Deftos, J. L., Dawson, B. F., Hogan, M. L. & Aurbach, G. D. (1971) *Proc. Nat. Acad. Sci. USA* 68, 63-67.
17. Potts, J. T., Jr., Keutmann, H. T., Niall, H. D., Habener, J. F., O'Riordan, J. L. H., Murray, T. M. & Aurbach, G. D. (1972) in *Endocrinology 1971. Proc. Third. Int. Symp.*, ed. Taylor, S. (Heinemann Medical Books Ltd. London, England), pp. 334-349.
18. Rasmussen, H., Sze, Y. L. & Young, R. (1964) *J. Biol. Chem.* 239, 2852-2857.
19. Brewer, H. B., Jr., Schlueter, R. & Aldred, P. (1970) *J. Biol. Chem.* 245, 5739-5742.
20. Arnaud, C. D., Tsao, H. S. & Littledike, E. T. (1971) *J. Clin. Invest.* 50, 21-34.
21. Smithies, O., Gibson, D., Fanning, E. M., Goodfiesh, R. M., Gilman, J. C. & Ballantyne, D. L. (1971) *Biochemistry* 10, 4912-4918.
22. Pisano, J. J. & Bronzert, T. (1969) *J. Biol. Chem.* 244, 5597-5607.
23. Pisano, J. J., Bronzert, T. & Brewer, H. B., Jr. (1972) *nat. Biochem.* 45, 43-59.
24. Hagenmaier, H., Ebbighausen, W., Nicholson, G. & Votsch, W. (1970) *Z. Naturforsch.* B25, 681-689.
25. Fairwell, T. & Lovins, R. E. (1971) *Biochem. Biophys. Res. Commun.* 43, 1280-1289.
26. Falas, H. M., Nagai, Y., Milne, G. W. A., Brewer, H. B., Jr., Bronzert, T. J. & Pisano, J. J. (1971) *Anal. Biochem.* 43, 288-299.
27. Arnaud, C. D., Goldsmith, R. S., Sizemore, G. W. & Oldham, S. B. (1972) *Med. Clin. N. Amer.* 56, 937-940.
28. Berson, S. A., Yalow, R. S., Aurbach, G. D. & Potts, J. T., Jr. (1963) *Proc. Nat. Acad. Sci. USA* 49, 613-617.
29. Reiss, E. & Canterbury, J. M. (1966) *Proc. Soc. Exp. Biol. Med.* 128, 501-504.
30. Potts, J. T., Jr., Murray, T. M., Peacock, M., Niall, H. D., Tregar, G. W., Keutmann, H. T., Powell, D. & Deftos, L. J. (1971) *Amer. J. Med.* 50, 639-649.
31. Habener, J. F., Segre, G. V., Powell, D., Murray, T. M. & Potts, J. T., Jr. (1972) *Nature New Biol.* 238, 152-154.
32. Canterbury, J. M. & Reiss, E. (1972) *Proc. Soc. Exp. Biol. Med.* 140, 1393-1398.
33. Canterbury, J. M., Levey, C. S. & Reiss, E. (1972) *J. Clinical Invest.*, in press.

The Amino-Acid Sequence of the Amino-Terminal 37 Residues of Human Parathyroid Hormone

(high-sensitivity automated Edman degradation/radio-iodination/peptide synthesis/immunoreactivity)

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ABSTRACT The sequence of the amino-terminal 37 residues of human parathyroid hormone has been established. The hormone used in these studies was isolated in highly purified form from parathyroid adenomata and was subjected to automated degradation in a Beckman sequencer. A high-sensitivity sequencing procedure employing ^{35}S -labeled phenylisothiocyanate of high specific activity as the coupling agent was used. The sequence obtained differs from that of bovine parathyroid hormone in three of the first 37 positions, and from that of porcine parathyroid hormone in two positions. A single human-specific residue was found (asparagine 16). The sequence obtained differs at three positions (22, 28, and 30) from the structure for human parathyroid hormone reported recently by Brewer *et al.* [(1972) *Proc. Nat. Acad. Sci. USA* 69, 3585-3588] and synthesized by Andreutta *et al.* [(1973) *Helv. Chim. Acta*, 56, 470-473]. We have carefully reviewed our data, reported here in detail, on the sequence positions in dispute. We must conclude, on the basis of all available data, that the structure that we propose is the correct structure. The objective resolution of these discrepancies in structural analysis through further chemical and immunochemical studies is important, since synthesis of human parathyroid hormone, in which there is widespread interest for physiological and clinical studies, must be based on the correct sequence of the human hormone if the peptide is to be genuinely useful.

Substantial advances have been made in recent years in our knowledge of parathyroid hormones, through studies of primary structure (1-3), structural requirements for biological activity (4, 5), biosynthesis (6-8), and metabolism (9-14). Most of these studies, including the development and application of radioimmunoassays capable of measuring plasma parathyroid hormone levels in man, have depended directly or indirectly upon the use of the bovine and porcine hormones. Purified human parathyroid hormone (HPTH), on the other hand, has been available in microgram quantities, sufficient only for limited studies of its chemical and immunological properties (15).

Recent improvements in extraction and isolation techniques, and the development of high-sensitivity methods for peptide sequence analysis have permitted us to determine the amino-acid sequence of the amino-terminal biologically active portion of HPTH (Fig. 1).

After the submission for publication in abstract form of our findings for the N-terminal 31 residues of HPTH (24), the report of Brewer *et al.* (29) of their own independent struc-

tural studies on HPTH was published. Marked discrepancies between the two structures, which differ in three of the first 30 residues, have prompted us to reexamine our data for each cycle of the several degradations performed with the phenylisothiocyanate method.

We now report in full the strategy and methods used in our sequence analysis as well as the quantitative aspects of the results and discuss the nature, implications, and possible approaches to resolution of the differences between the findings of Brewer *et al.* (29) and ourselves concerning the sequence of the amino-terminal portion of human parathyroid hormone.

MATERIALS AND METHODS

The HPTH used in these studies was extracted from 500 g of pooled human adenoma tissue by use of 88% phenol, followed by treatment with 6% NaCl and precipitation with trichloroacetic acid (15, 16). The hormone was further purified by gel filtration on Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.) and ion-exchange chromatography on carboxymethyl-cellulose (Whatman CM-52; Reeve Angel Co., Summit, N.J.) (16). Hormone purification was monitored by radioimmunoassay (11).

Automated Edman degradations were performed on the Beckman model 890 sequencer (Beckman Instruments, Palo Alto, Calif.) using the single-coupling, double-cleavage method of Edman and Begg (17), and other procedures recently described (18). Manual Edman degradations were performed as previously described (19). Reagents and solvents were obtained from Beckman Instruments. ^{35}S -labeled phenylisothiocyanate was obtained from Amersham/Searle (Arlington Heights, Ill.).

The phenylthiohydantoin (PTH) derivatives were identified by thin-layer chromatography (TLC) on silica gel plates (Analtech, Inc., Newark, Del.) (17, 20) and by gas-liquid chromatography (21) using a two-column system (10% DC-500 and 1.5% AN-600). PTH-histidine was identified by the Pauly reaction (22) and PTH-arginine by the phenanthrene-quinone reaction (23). Quantitative yields of the PTH-amino-acid derivatives at each cycle of degradation were determined by comparison with known standards on gas-liquid chromatography. The ^{35}S -PTH-amino acids were separated by TLC; the radioactive spots were identified by autoradiography and quantitated in a Packard model 3375 liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Mono-iodohistidine (MIH) and di-iodohistidine (DIH) were synthesized by the method of Brunnings (25) using the modifications of Savoie *et al.* (26). The phenylthiohydantoin

Abbreviations: HPTH, human parathyroid hormone; PTH, phenylthiohydantoin; TLC, thin-layer chromatography; MIH, mono-iodohistidine; DIH, di-iodohistidine.

TABLE 1. Automated Edman degradation of native HPTH

PTH-amino acids			PTH-amino acids		
Cycle	Found	Yield (nmol)	Cycle	Found	Yield (nmol)
1	Ser	80.0	21	Val	23.8
2	Val	130.2	22	Glu†	10.5
				Thr	7.3
	Ser	85.3	23	Trp	10.1
	Glu	71.4	24	Leu	18.7
	Ile	108.6	25	Arg	13.0
	Glu*	20.7	26	Lys	14.6
	Gln	35.0			
	Leu	97.5	27	Lys	15.9
	Met	51.5	28	Leu	11.0
	His	42.8	29	Glu*	3.1
				Gln	2.8
	Asp*	30.6	30	Asp	4.5
	Asn	20.1			
	Leu	97.5	31	Val	9.1
	Gly	47.5	32	Ser†	0.7
	Lys	62.1	33	Asp*	2.7
				Asn	‡
	His	27.6	34	Phe	3.0
	Leu	53.5	35	Val	3.0
	Asp*	17.7	36	Ala	1.4
	Asn	18.1			
1	Ser	43.0	37	Ileu	2.1
1	Met	24.8	38	—	—
1	Glu	20.2	39	Ala	1.2
2	Arg	16.4	40	—	—

* Found deamidation during the conversion reaction accounts for the presence of the free acid as well as the amide form at positions 29 (glutamines) and positions 10, 16, and 33 (asparagines).

† Ser† for discussion.

‡ The identification.

Derivatives of MIH and DIH were prepared as described by Edman (27). PTH-MIH and PTH-DIH were separated from all other PTH-amino-acid derivatives by TLC in the solvent system *n*-butyl acetate:water:propionic acid:formamide (240:200:80:60). PTH-[¹²⁵I]MIH and PTH-[¹²⁵I]DIH were identified by cochromatography with their respective [¹²⁵I] derivatives followed by autoradiography, and quantitated by counting in a Packard model 3001 gamma well spectrometer.

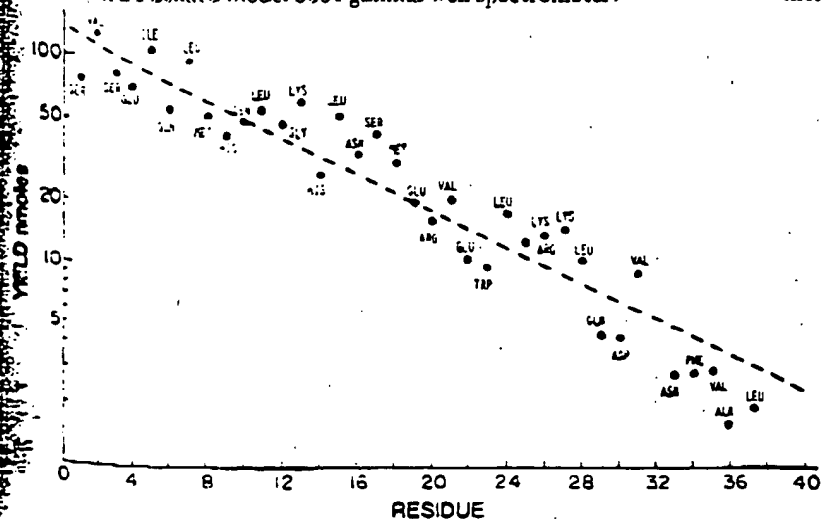


FIG. 2. Yields of phenylthiohydantoin-amino acids obtained during automated degradation of native human parathyroid hormone. See Table 1 and text.

NH₂-SER-VAL-SER-GLU-ILE-GLN-LEU-MET-HIS-ASN-LEU-GLY-LYS-HIS-LEU-ASN

SER 27

MET 18

GLU 19

ARG 20

VAL 21

...LEU-ALA-VAL-PHE-ASN-HIS-VAL-ASP-GLN-LEU-LYS-LYS-ARG-LEU-TRP-GLU

FIG. 1. The amino-terminal 37 residues of human parathyroid hormone.

Cleavage of the hormone with cyanogen bromide (CNBr) was carried out in 70% formic acid for 12 hr, 20°, with a 100-fold molar excess of CNBr. Digestion with TPCK-trypsin (Worthington Biochemical Corp., Freehold, N.J.) was performed in 0.2 M trimethylamine acetate buffer (pH 9.2) at 37° for a period of 2 hr using an enzyme-to-substrate ratio of 1/100.

RESULTS

Purified HPTH (140 nmol) was subjected to automated Edman degradation for 40 cycles. The PTH-amino acid derivatives identified at each cycle of this degradation, and their yields, are presented in Table 1. To illustrate repetitive yield these results are also plotted in Fig. 2.

As shown in Table 1, unique amino-acid assignments, and quantitation of the single residue identified, were possible at all but two of the first 37 cycles of this degradation. At cycle 22, evidence was obtained for two residues, threonine and glutamic acid. Since the quantitative recovery of both of these residues can be low, further experiments were performed prior to definitive assignment of position 22. At cycle 32, a rise in PTH-serine above background levels was observed. However, its yield (Table 1) was considerably below that expected, even for the labile phenylthiohydantoin derivative of serine (18). Although histidine is present at this position in porcine and bovine parathyroid hormones, this residue could not be detected either by the Pauly method or by a definite increase in radioactivity associated with [³⁵S]PTH-histidine at this cycle. However, since the overall yield at this stage of the degradation was near the detection limits for histidine by these methods, further experiments were performed prior to assignment of this position.

The presence of methionine at positions 8 and 18 of the native hormone accounted for both methionines found by amino-

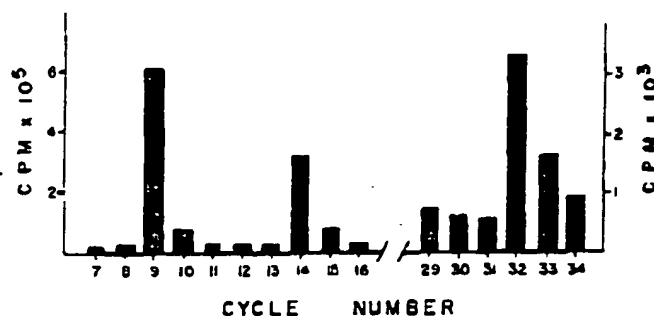


FIG. 3. Release of radioactive (^{125}I -labeled) phenylthiohydantoin derivatives of mono- and di-iodohistidine during automated degradation of ^{125}I -labeled human parathyroid hormone. The break in the horizontal axis is introduced to simplify the presentation. No significant release of histidine-associated radioactivity was seen in cycles 17-28. [^{125}I]Histidine was found at cycles 9, 14, and 32, indicating the presence of histidine at these positions. See text. Numbers on the ordinates are to be multiplied by the indicated factors to obtain the experimental values.

acid analysis (10). This indicated that cleavage of the human hormone with CNBr should result in generation of three principal peptide- representing residues 1-8, 9-18, and 19-carboxyl terminus- of the native hormone. HPTH (27 nmol) was cleaved with CNBr and the unfractionated peptide mixture subjected to 19 cycles of Edman degradation. The results are presented in Table 2. The expected three end-groups, Ser¹, His⁹, and Glu¹⁹ were identified at cycle one of the degradation. At cycle 4 of the degradation, corresponding to residues 4, 12, and 22 of the intact hormone, only PTH-Glu and PTH-Gly were observed in significant yield. No threonine was detected at this cycle. Therefore, glutamic acid was assigned as residue 22 of native HPTH. The significance of the finding of threonine in the amino-terminal degradation remains uncertain. As can be seen in Table 2, the results of the CNBr mixture analysis also provided complete confirmation of all residue assignments made on the basis of the amino-terminal degradation on intact HPTH.

Since the limited supply of purified HPTH excluded the use of conventional protein chemical methods for reexamination of position 32, an alternative radioactive micro-method was developed to permit detection of histidine residues. Purified HPTH (0.75 μg) was iodinated with ^{125}I by a modification of the Hunter-Greenwood procedure (28). Unlabeled bovine parathyroid hormone was then added as carrier and the mixture was degraded in the sequencer. At each cycle, the radioactivity migrating with PTH-[^{125}I]MIH and PTH-[^{125}I]DIH on TLC was determined. These data (Fig. 3) demonstrate

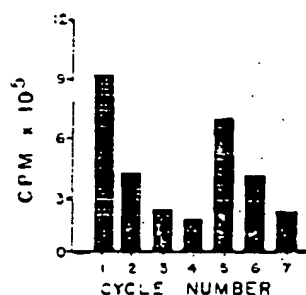


FIG. 4. Release of radioactive (^{125}I -labeled) phenylthiohydantoin derivatives of mono- and di-iodohistidine during degradation of a tryptic digest of iodinated HPTH. [^{125}I]Histidine was released at cycles 1 and 5. See text.

TABLE 2.

Cycle	CNBr1	CNBr2	CNBr3	Yield of PTH Derivative (nmol)
1	Ser ¹	His ⁹	Glu ¹⁹	Ser 15.3, His ⁹ , Gly 27.0
2	Val	Asn	Arg	Val 11.7, Asn 4.3, Arg†
3	Ser	Leu	Val	Ser 10.2, Leu 8.5, Val 18.4
4	Glu	Gly	Glu	Glu 4.9, Gly 3.1
5	Ile	Lys	Trp	Ile 10.2, Lys‡, Trp 58
6	Gln	His	Leu	Gln 4.9, His ⁹ , Leu 10.5
7	Leu	Leu	Arg	Leu 9.8, Arg†
8	Met‡	Asn	Lys	Met 3.4, Asn‡, Lys‡
9	His	Ser	Lys	His ⁹ , Ser 4.2, Lys‡
10	Asn	—	Leu	Asn‡, Leu 7.5
11	Leu	—	Gln	Leu 1.9, Gln 4.6
12	Gly	—	Asp	Gly 0.9, Asp 2.3
13	—	—	Val	Val 4.2
14	—	—	—	—
15	—	—	Asn	Asn‡
16	—	—	Phe	Phe 2.6
17	—	—	Val	Val 4.5
18	—	—	Ala	Ala 2.0
19	—	—	Leu	Leu 2.0

* Identification by Pauly reaction.

† Identification by phenanthrenequinone reaction.

‡ Identification by thin-layer chromatography.

§ Presence of methionine at cycle 8 with the following four residues obtained at cycles 9-12 indicates that cleavage of the Met⁸-His⁹ bond by cyanogen bromide was incomplete. Residues from this sequence, presumably representing the 1-18 peptide fragment, could not be detected subsequent to cycle 12.

* Histidine, subsequently found to occupy position 32 (see text) was not detected at the expected cycle (number 14) of this degradation.

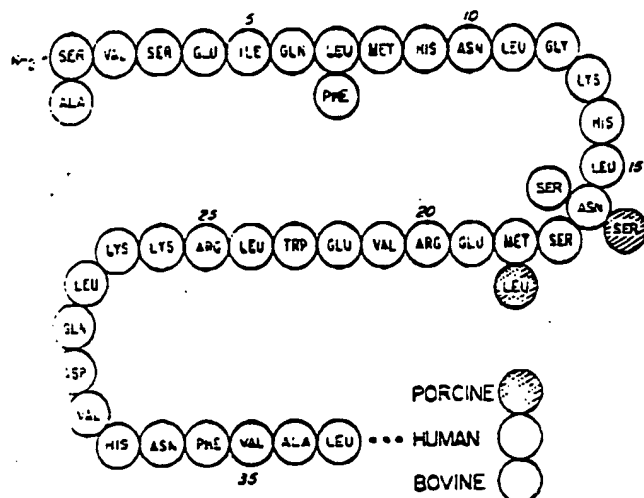
the presence of histidine at cycle 32, and confirm the histidine at cycles 9 and 14.

To confirm these results further, and in particular to examine the differences between Brewer *et al.* (29) and ourselves concerning the nature of residue 28, iodinated HPTH was digested with trypsin and then subjected to Edman degradation for seven cycles. The PTH derivatives of [^{125}I]MIH and [^{125}I]DIH were found at cycles 1 and 5 of the degradation (Fig. 4). The histidine at cycle 1 further confirmed the Lys¹²-His¹⁴ sequence already determined. The finding of histidine at cycle 5 would be predicted on the basis of tryptic cleavage carboxyl to Lys²⁷, and therefore both supports the assignment of His³² and argues against the report of Brewer *et al.* (29) that residue 28 is lysine.

DISCUSSION

The amino-terminal sequence we propose for HPTH differs from that of both the bovine and the porcine hormones. HPTH differs from bovine parathyroid hormone (Fig. 5) at positions 1, 7, and 16. The porcine and human hormones differ at positions 16 and 18. Asn¹⁶ is the only unique residue found in the active region of human parathyroid hormone.

Unexpectedly, our structure differs at three positions from that recently proposed by Brewer *et al.* (29) for the amino-terminal 34 residues of HPTH. They report residue 22 to be glutamine, residue 28 to be lysine, and residue 30 to be leucine. If correct, all these changed residues would be unique to the human hormone. In contrast, we find residue 22 to be glutamic



5. Comparison of the amino-terminal sequences of porcine, and human parathyroid hormones. The central sequence is that of the human hormone (residues 1, 7, and 25). Residues differing in the bovine hormone are stippled; those in porcine parathyroid hormone (16 and 18) are hatched.

Residue 28 to be leucine, and residue 30 to be aspartic acid. The residues we have identified are identical with those at corresponding positions in both the porcine and bovine hormones.

Both groups have isolated the hormone from essentially similar sources, i.e., human adenoma tissue pooled from multiple donors. The possibility that there are two hormonal forms which differ as markedly as those of the two proposed sequences is highly unlikely.

We have carefully reexamined our data from both degradations with particular emphasis on the positions in question. In our degradation was any glutamine observed at cycles corresponding to residue 22. Glutamine and asparagine can undergo deamidation during degradation. However, in both degradations (Tables 1 and 2) glutamine and asparagine residues were detected at cycles beyond position 22, making it implausible that the glutamic acid detected at position 22 was originally glutamine. At cycles corresponding to residues 28 and 30, leucine and aspartic acid were clearly identified by their predominant yields (Tables 1 and 2). The relative rise in yield of these residues above and subsequent fall to background levels is shown in Fig. 6.

In automated Edman degradation the phenomenon of increasing overlap, which tends to be cumulative from cycle to cycle, has been well documented (17, 18, 30). Edman and Begg have, however, found that use of a double-cleavage program can limit this overlap to relatively low levels (17) even in extended degradations. In our amino-terminal degradation, which employed such a double-cleavage program, overlap rose from 4.5% at cycle 12 to 14.5% at cycle 28. Brewer *et al.* reported quantitative data only at position 12; their data permit the calculation that there was a 32% overlap at this early phase of degradation. The natural increase of this already substantial overlap, particularly in view of their use of a single-cleavage program, would make assignment of repeating residues at later cycles of the degradation, such as the putative Lys²⁸ in a sequence Lys²⁴-Lys²⁷-Lys²⁸, particularly hazardous.

If, as proposed by Brewer *et al.* (29), residue 25 is lysine, tryptic digestion of [¹²⁵I]HPTH would lead to cleavage of the

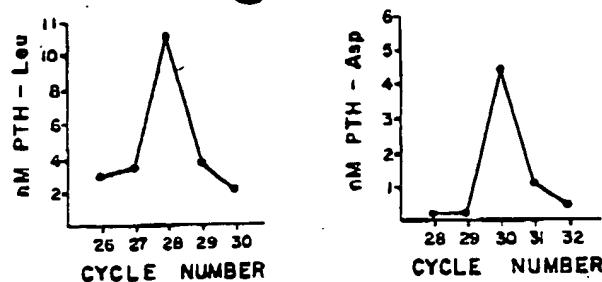


Fig. 6. Yields of PTH-leucine at cycles 26-30 and of PTH-aspartic acid at cycles 28-32 obtained during automated degradation of native human parathyroid hormone. A sharp rise above background levels is seen at cycle 28 for leucine and at cycle 30 for aspartic acid. See text.

hormone carboxyl-terminal to residue 28. Therefore, the PTH derivatives of [¹²⁵I]MIH and [¹²⁵I]DIH corresponding to His²⁵ would be released at the fourth cycle of degradation, rather than the fifth. Clearly, however, histidine is released only at the first and fifth cycles (Fig. 4).

Ultimate resolution of the differences in the proposed structures must await further studies. However, a comparison of our results and methods with the published results and methods of Brewer *et al.* (29) leads us to conclude that our proposed structure based on a variety of approaches is more likely to be correct.

Tests of the biological and immunological properties of synthetic peptides corresponding to our structure and to the structure proposed by Brewer *et al.* (29) for the amino-terminal portion of human parathyroid hormone may prove helpful in objectively resolving the discrepancies in the structures proposed. The marked differences, which include a change in net charge of three within a sequence of nine residues, might affect biological activity. Even more likely is the possibility that such charge differences will result in clear-cut differences in immunoreactivity when the synthetic peptides based on the two proposed structures are each compared with native human parathyroid hormone in their ability to combine with antisera directed against the amino-terminal region (11).

If the structure of Brewer and associates is, as we believe, incorrect, use of antisera generated against the corresponding synthetic peptide for radioimmunoassay studies could confuse rather than aid attempts to more accurately measure HPTH or to understand the complex pattern of metabolism of parathyroid hormone (11). A preliminary immunoassay study based on the peptide of Brewer *et al.* (29) and Andreatta *et al.* (34) has already been published (35). Clearly it is extremely important to establish whether our sequence or that of Brewer *et al.* (29) represents the native HPTH structure before various laboratories embark on extensive immunological studies using synthetic HPTH peptide.

An amino-terminal tetratriacontapeptide based on the structure proposed here has been synthesized by the solid-phase method (31). Studies of the potency of this peptide as measured *in vitro* by activation of renal-cortical adenylate cyclase indicate that its activity is 1030 units/mg, closely equivalent, on a molar basis, to the potency of 350 units/mg (16) for native human parathyroid hormone in this assay. Assays *in vivo* using the chick hypercalcemia assay (32) indicate a potency of 7000 units/mg, an activity identical to that of the bovine peptide 1-34 (no native human parathyroid hormone was available for assay in this system).

The immunological activity of the synthetic peptide has been examined with several antisera directed against the amino-terminal region of parathyroid hormone. Tests against antiserum 199 (23) and GP-1 (11) indicated that reactivity on a molar basis of our synthetic peptide was identical qualitatively and quantitatively to that of the native human hormone. No details have been reported on the specific biological or immunological activity of the synthetic peptide of Andreatta et al. (34), whose structure was based on the structure reported by Brewer et al. (29). Comparisons based on detailed biological and immunological tests of the two synthetic peptides in various laboratories should be of considerable interest.

Our present findings carry several implications for the structural and comparative immunochemical studies of human parathyroid hormone. The sequence of HPTH we find is identical with that of either the bovine or porcine hormone at 36 of the first 37 residues; the changes found do not affect net charge and do not greatly alter physicochemical properties. Hence, although some improvements in detection of human parathyroid hormone might result from use of antisera directed against the amino-terminal sequence of the human hormone, the improvements, in our view, might not be large. In fact, the success encountered already in numerous laboratories in detection of the human hormone with immunoassays based on the bovine molecule is consistent with the overall chemical similarity found in the amino-terminal sequences of the three species of parathyroid hormone.

On the other hand, previous immunochemical and analytical evidence (11, 15, 16) indicates that more marked differences in structure between bovine and human hormones are likely to be found in the carboxyl-terminal region. Since a large carboxyl-terminal fragment appears to be the major form of immunoreactive parathyroid hormone in the human circulation (11), antisera that recognize the carboxyl end of the human hormone are most likely to significantly improve immunoassay sensitivity. Further sequence studies on HPTH, followed by synthesis of selected peptides from the carboxyl-terminal two-thirds of the molecule, may well result in antisera considerably more sensitive for detection of human parathyroid hormone.

Collection of the human parathyroid adenomata used in this study was made possible through the cooperation of many individuals and institutions in the United States, Canada, and overseas. Special thanks are due the Medical Research Council of Great Britain for help with this project. This investigation was supported in part by Grants AM 11794 and AM 04501 from the National Institute of Arthritis, Metabolic and Digestive Diseases. G.V.S. is the George Morris Piersol Teaching and Research Scholar of the American College of Physicians and Special Fellow of the National Institute of Arthritis, Metabolism and Digestive Diseases.

- Niall, H. D., Keutmann, H. T., Sauer, R., Hogan, M. L., Dawson, B. F., Aurbach, G. D. & Potts, J. T., Jr. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* 351, 1586-1588.
- Brewer, H. B., Jr. & Ronan, R. (1970) *Proc. Nat. Acad. Sci. USA* 67, 1862-1869.
- O'Riordan, J. L. H., Woodhead, J. S., Robinson, C. J., Parsons, J. A., Keutmann, H. T., Niall, H. D. & Potts, J. T., Jr. (1971) *Proc. Roy. Soc. Med.* 64, 1263-1265.
- Keutmann, H. T., Dawson, B. F., Aurbach, G. D. & Potts, J. T., Jr. (1972) *Biochemistry* 11, 1973-1979.
- Potts, J. T., Jr., Tregear, G. W., Keutmann, H. T., Niall, H. D., Sauer, R., Deftos, L. J., Dawson, B. F., Hogan, M. L. & Aurbach, G. D. (1971) *Proc. Nat. Acad. Sci. USA* 68, 63-67.
- Tregear, G. W., van Rietschoten, J., Greene, E., Keutmann, H. T., Niall, H. D., Reit, B., Parsons, J. A. & Potts, J. T., Jr. (1973) *Endocrinology* 93, 1349-1353.
- Cohn, D. V., MacGregor, R. R., Chu, L. L. H. & Hamilton, J. W. (1972) in *Calcium, Parathyroid Hormone and the Calcitonins* eds. Talmage, R. V. & Munson, P. L. (Excerpta Medica, Amsterdam, The Netherlands), pp. 173-182.
- Kempner, B., Habener, J. F., Potts, J. T., Jr. & Rich, A. (1972) *Proc. Nat. Acad. Sci. USA* 69, 643-647.
- Habener, J. F., Powell, D., Murray, T. M., Mayer, G. P. & Potts, J. T., Jr. (1971) *Proc. Nat. Acad. Sci. USA* 68, 2986-2991.
- Potts, J. T., Jr., Niall, H. D., Tregear, G. W., Van Rietschoten, J., Habener, J. F., Segre, G. V. & Keutmann, H. T. (1973) *Mt. Sinai J. Med.* 40, 448-461.
- Segre, G. V., Habener, J. F., Powell, D., Tregear, G. W. & Potts, J. T., Jr. (1972) *J. Clin. Invest.* 51, 3163-3172.
- Canterbury, J. M. & Reiss, E. (1972) *Proc. Soc. Exp. Biol. Med.* 140, 1393-1398.
- Canterbury, J. M., Levey, C. S. & Reiss, E. (1973) *J. Clin. Invest.* 52, 524-527.
- Goldsmith, R. S., Furszyfer, J., Johnson, W. J., Fournier, A. E., Sizemore, G. W. & Arnaud, C. D. (1973) *J. Clin. Invest.* 52, 173-180.
- O'Riordan, J. L. H., Aurbach, G. D. & Potts, J. T., Jr. (1971) *Endocrinology* 89, 234-239.
- Keutmann, H. T., Barling, P. M., Hendy, G. N., Segre, G. V., Niall, H. D., Aurbach, G. D., Potts, J. T., Jr. & O'Riordan, J. L. H. (1974) *Biochemistry*, in press.
- Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* 1, 80-91.
- Niall, H. D. (1973) in *Methods in Enzymology*, eds. Hirs, C. H. W. & Timasheff, S. N. (Academic Press, New York), Vol. XXVII, part D, in press.
- Niall, H. D., Keutmann, H. T., Copp, D. H. & Potts, J. T., Jr. (1969) *Proc. Nat. Acad. Sci. USA* 64, 771-778.
- Morgan, F. J. & Henschen, A. (1969) *Biochim. Biophys. Acta* 181, 43-104.
- Pisano, J. J. & Bronzert, T. (1969) *J. Biol. Chem.* 244, 5597-5607.
- Easley, C. W. (1965) *Biochim. Biophys. Acta* 107, 386-388.
- Yamada, S. & Itano, H. A. (1968) *Biochim. Biophys. Acta* 130, 538-540.
- Jacobs, J. W., Sauer, R. T., Niall, H. D., Keutmann, H. T., O'Riordan, J. L. H., Aurbach, G. D. & Potts, J. T., Jr. (1973) *Fed. Proc.* 32, 2445.
- Brunnings, K. J. (1917) *J. Amer. Chem. Soc.* 69, 205.
- Savoie, J. C., Thomopoulos, P. & Savoie, F. (1973) *J. Clin. Invest.* 52, 106-115.
- Edman, P. (1950) *Acta Chem. Scand.* 4, 277-282.
- Hunter, W. M. & Greenwood, F. C. (1962) *Nature* 194, 495-498.
- Brewer, H. B., Jr., Fairwell, T., Ronan, R., Sizemore, G. W. & Arnaud, C. D. (1972) *Proc. Nat. Acad. Sci. USA* 69, 3585-3588.
- Hermanson, M. A., Ericson, L. H., Titani, K., Neurath, H. & Walsh, K. A. (1972) *Biochemistry* 11, 4493-4502.
- Tregear, G. W., van Rietschoten, J., Greene, E., Keutmann, H. T., Niall, H. D., Parsons, J. A. & Potts, J. T., Jr. (1974) "Principles and recent applications in the solid-phase synthesis of peptide hormones," in *Endocrinology 1973: Proceedings of the Fourth International Symposium*, ed. Taylor, S. (Heinemann, London), in press.
- Parsons, J. A., Reit, B. & Robinson, C. J. (1973) *Endocrinology* 92, 454-462.
- O'Riordan, J. L. H., Woodhead, J. S., Addison, G. M., Keutmann, H. T., & Potts, J. T., Jr. (1972) "Localization of Antigenic Sites in Parathyroid Hormone," in *Endocrinology 1971: Proceedings of the Third International Symposium* ed. Taylor, S. (Heinemann, London), pp. 386-392.
- Andreatta, R. M., Hurrmann, A., Johl, A., Kamber, B., Maier, R., Riniker, B., Rittel, W. & Sieber, P. (1973) *Helv. Chim. Acta* 56, 470-473.
- Arnaud, C. D., Brewer, H. B., Rittel, W., Bordier, P. & Goldsmith, R. S. (1973) *The Endocrine Society, 55th Annual Meeting, Chicago, 1973*, Abstract no. 222.

Isolation of Human Parathyroid Hormone†

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ABSTRACT: A pure preparation of human parathyroid hormone has been isolated using as starting material 500 g of pooled gland tissue removed at surgery. Following initial extraction of the acetone-dried parathyroid tissue by means of phenol, a trichloroacetic acid precipitate was obtained which contained 4% hormone by radioimmunoassay. Further purification of the initial CCl_3COOH precipitate was achieved by polyacrylamide gel filtration followed by a final step of ion-exchange chromatography. Repeat extractions of the original tissue residue provided an additional yield of hormone, although the hormone content of most of these CCl_3COOH preparations was lower. Additional ion-exchange chromatographic steps were needed for final purification of hormone derived from these repeat tissue extractions. The purified human hormone preparations from the various extractions

were, however, found to be identical chemically and immunologically. An overall yield of 3.2 mg of purified hormone suitable for structural studies was obtained, sufficient for sequence analysis of the biologically active amino-terminal portion of the molecule. The biological activity by *in vitro* renal adenylyl cyclase assay is 350 units/mg, considerably lower than that of the hormone from the bovine species. The amino acid composition of human parathyroid hormone bears considerable resemblance to that of bovine and of porcine hormone. There are, however, a number of compositional differences which have not been accounted for in the sequence of the amino-terminal region. This indicates that when structural studies of the carboxyl-terminal portion are undertaken, they will reveal several sequence positions which are unique to the human hormone.

Evidence from several laboratories suggests that the state of parathyroid hormone in the circulation, especially in certain disease states, may be very complex (Habener *et al.*, 1971; Canterbury and Reiss, 1972; Segre *et al.*, 1972; Goldsmith *et al.*, 1973; Silverman and Yalow, 1973). Application of the radioimmunoassay to measurement of parathyroid hormone in the peripheral circulation has demonstrated the presence of multiple fragments in addition to the intact hormone. The possible contribution of large precursor forms (Cohn *et al.*, 1972; Habener *et al.*, 1972) to the spectrum of immunoreactive hormone may further complicate this picture.

Attempts to clarify the nature of circulating human hormone and to ascertain the significance of the various hormonal fragments have thus far relied principally on radioimmunoassay systems and structure-function studies based on the bovine and porcine hormones. These problems have emphasized the need for knowledge of the chemical structure of human parathyroid hormone (HPTH).¹

The structural analyses previously carried out on the hormone from bovine (Brewer and Ronan, 1970; Niall *et al.*, 1970) and porcine (O'Riordan *et al.*, 1971a) species benefitted from the availability of gland tissue obtained from slaughter-

house sources. The study of human parathyroid hormone (Arnaud *et al.*, 1970; O'Riordan *et al.*, 1971b) has been limited by the extremely small quantities of available starting material: parathyroid tissue removed at surgery. Using this source, O'Riordan *et al.* (1971b) isolated 0.5 mg of purified hormone sufficient for preliminary immunological and chemical characterization. It was observed, however, that throughout the isolation procedure the recoveries of hormone were considerably lower than those obtained during isolation of bovine or porcine hormone. These problems have stressed the need not only for efficient extraction and purification procedures, but also for the application of precise and economical methods for monitoring recoveries and purity throughout the isolation.

Recently, accumulation of sufficient quantities of human parathyroid tissue has permitted the resumption of efforts to characterize the human hormone and as a result, sequence analysis of the biologically active amino-terminal region has been reported by two groups of investigators (Brewer *et al.*, 1972; Niall *et al.*, 1974). To be described here is our procedure for the extraction and purification of hormone from 500 g of human gland tissue, using several modifications designed to improve recoveries. In the course of the work, compositional analyses of the hormone and an assessment of its biological activity have also been carried out.

The purified hormone obtained was sufficient for sequence determination of the amino-terminal 37 residues of the molecule (Niall *et al.*, 1974). Since the structural analysis of the entire carboxyl-terminal portion remains to be done, the information described in this report should be useful in planning and carrying out future hormone purifications when sufficient tissue again becomes available.

Materials and Methods

Parathyroid Tissue. The human parathyroid tissue used consisted predominantly of adenomas but also included hyperplastic tissue from patients with primary and secondary

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Abbreviations used are: HPTH, human parathyroid hormone; BPTH, bovine parathyroid hormone; PPTH, porcine parathyroid hormone; CMC, carboxymethylcellulose; MRC, Medical Research Council.

hyperparathyroidism. This material was obtained through the cooperation of a large number of medical centers in the United Kingdom, United States, and several European countries. The tissue was frozen on dry ice immediately upon surgical removal, and stored at -20° or below until used.

Extraction Procedure. The procedure followed for extraction of a crude hormone preparation from the pooled gland tissue is outlined in Figure 1. The tissue was first homogenized in acetone at -20° and filtered. The residue was defatted by homogenization in hexane, followed by filtration and washing with acetone, all at -20° . The resulting powder (AP, Figure 1) was kept overnight in an evacuated desiccator, and processed into a trichloroacetic acid precipitate (CCl_3COOH -HPTH) after extraction with phenol, by means of a procedure derived from that described originally by Aurbach (1959).

The acetone powder was suspended in 1 l. of 90% phenol containing 0.5% 2-mercaptoethanol. The suspension was diluted out with 5 l. of 20% acetic acid in acetone, 13 ml of 4 M sodium chloride was added and, after standing for 1.5 hr, the suspension was filtered in the presence of Celite.

The residue was saved and ultimately subjected to repeat extractions, as outlined in the Results section and in Figure 1. The filtrate was treated by gradual addition of ether and collection of the resulting precipitate (AE, Figure 1). The ether powder was resuspended in glacial acetic acid in the presence of cysteine-HCl, diluted with water, and centrifuged. The precipitate was saved for reprocessing later while the supernatant was made to 6% (w/v) with sodium chloride.

The precipitate formed upon addition of the salt was separated by centrifugation, resuspended, and again treated with sodium chloride. The combined salt supernatants (AS, Figure 1) were made to 4% in trichloroacetic acid and the resulting suspension was centrifuged. The CCl_3COOH precipitate (AT, Figure 1) was resuspended in acetic acid and the CCl_3COOH removed by addition of IRA-400 acetate resin (Rohm and Haas, Darmstadt, Germany). When the solution had completely cleared, it was passed through a 1.5×20 cm column of IRA-400 and the eluate was lyophilized.

Measured aliquots were removed at successive stages of the extraction (AE, AS, AT; Figure 1) for radioimmunoassay, and a weighed aliquot of the acetone powder starting material (AP, Figure 1) was also retained for assays to assess overall recovery.

A 20-mg aliquot of CCl_3COOH precipitate from the initial extraction (AT-1) was also retained for calibration against the purified hormone, for use as a future radioimmunoassay standard for HPTH.

Gel Filtration. A 2.5×130 cm column of Bio-Gel P-100 (Bio-Rad, Riverside, Calif.) was equilibrated with 0.14 M ammonium acetate (pH 4.9) at 4° . The column was presaturated by application of 500 mg of a CCl_3COOH precipitate of bovine parathyroid hormone (BPTH) containing about 50 mg of hormone. The BPTH was eluted with the same buffer and then 2 l. (four column volumes) of buffer was allowed to pass through the column before application of any HPTH.

A flow rate of 20 ml/hr was used in all runs. Optical density of eluate tubes was read at 250 and 280 m μ using the Beckman DB-G spectrophotometer (Beckman Instruments, Fullerton, Calif.). Elution of hormone was defined by radioimmunoassay, and the appropriate tubes pooled and lyophilized twice.

Ion-exchange chromatography was carried out using a 0.9×10 cm column of carboxymethylcellulose (CMC) (Whatmar CM-52; Reeve-Angel, Inc., Clifton, N. J.). Prior to use for the human hormone, the CMC column was presaturated by

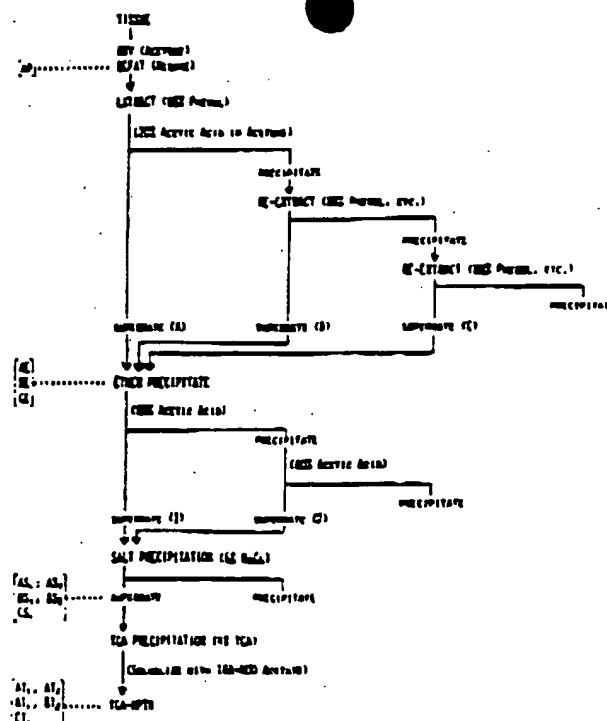


FIGURE 1: Outline of procedure for extraction of parathyroid hormone from pooled human gland tissue. Code designations for various reextracts of the phenol residue and ether powder (described in the text) appear along left margin.

chromatography of 20 mg of partially purified (Sephadex grade) bovine parathyroid hormone. After elution of the BPTH with high ionic strength buffer, the column was exhaustively reequilibrated with the starting buffer, 0.01 M ammonium acetate (pH 4.9, conductivity 0.6 mmho). The lyophilized human hormone pool from P-100 chromatography was applied in the same buffer, and a linear gradient of increasing conductivity established at 4° by means of a Varigrad gradient maker (Buchler Instruments, Fort Lee, N. J.), using 0.2 M ammonium acetate (pH 6.0, conductivity 12.5 mmhos) as second buffer. The most satisfactory linear gradient was achieved by use of two chambers (each 90 ml) of starting buffer and a third chamber (also 90 ml) of second buffer. Following completion of the gradient, 1 M ammonium acetate (pH 6.5, conductivity 30 mmhos) was allowed to run through the column. All conductivity measurements were taken at 20° using the Radiometer (Copenhagen) Model CDM-2c conductivity meter.

For CMC chromatography employing buffers in 8 M urea, the buffer constituents were added to the freshly deionized urea immediately before chromatography, which was carried out at room temperature. Column dimensions and buffer parameters were otherwise the same as described above.

Radioimmunoassays. Recoveries of hormone at the initial stages of extraction were assessed by use of the immunoradiometric assay as described by Addison *et al.* (1971) using ^{125}I -labeled antibodies extracted from guinea pig 199, an anti-bovine antiserum (O'Riordan *et al.*, 1972). The reference preparation was a CCl_3COOH precipitate prepared earlier (O'Riordan *et al.*, 1971b), designated HT-67. Radioiodine was purchased from Amersham Ltd. (Amersham, England).

The hormone preparations AE, AS, and AT (Figure 1) were diluted and added directly to the assay. Aliquots of the acetone powder starting material (AP, Figure 1), in amounts of 10-30

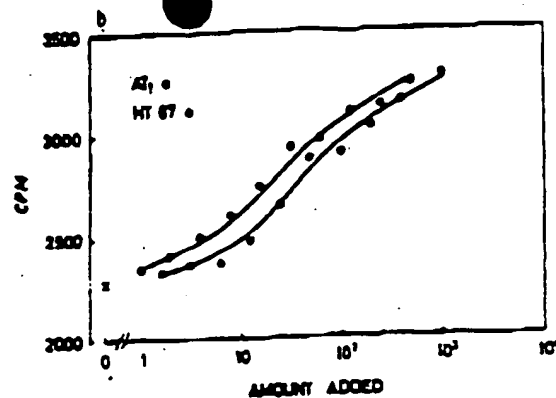
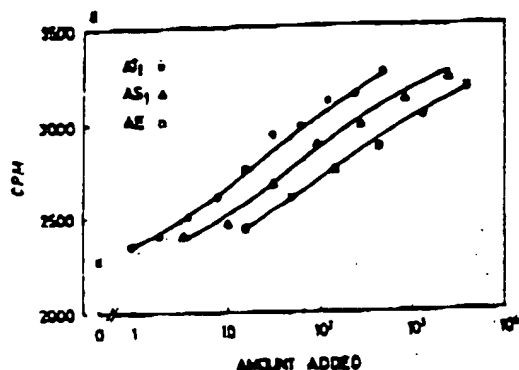


FIGURE 2: (a) Comparison of response curves for human parathyroid hormone aliquotted from successive stages of initial extraction, in an immunoradiometric assay using ^{125}I -labeled GP-199 antiserum (Addison *et al.*, 1971): AE, ether powder; AS₁, salt supernatant; AT₁, CCl_3COOH precipitate. Ordinate represents counts of hormone-bound, labeled antibody remaining in supernatant after precipitation of unbound antibody with solid-phase immunosorbant. Abscissa represents increasing volume aliquots of the respective extracts added into the assay. (b) Comparison of immunoassay response curves for CCl_3COOH -HPTH preparation AT-1 from current purification and CCl_3COOH -HPTH preparation HT-67 previously prepared by O'Riordan *et al.* (1971b). Assay conditions and coordinates are as described for part a.

mg, were extracted either with 20% acetic acid in 8 M urea or with 90% phenol (2 ml, 18 hr, room temperature for either method) prior to immunoassay.

Recovery studies during the steps of purification subsequent to the CCl_3COOH stage were carried out using a modification (Segre *et al.*, 1972) of the standard radioimmunoassay as developed for bovine parathyroid hormone by Berson *et al.* (1963). Guinea pig 1 antiserum, also an anti-bovine preparation (O'Riordan *et al.*, 1969) was used at a dilution of 1:300,000. The same CCl_3COOH -HPTH standard (HT-67) was used as a reference in these assays. ^{125}I for labeling of purified BPTH tracer was obtained from New England Nuclear (Wilmington, Mass.).

Scanning of the column profiles for location of eluted hormone was performed using a shortened assay procedure. Guinea pig 1 antiserum was used at a dilution of 1:50,000, enabling incubations to be carried out overnight instead of the usual 3 days. In these assays 20- μl aliquots from selected tubes were assayed in three successive 50-fold dilutions.

Edman Degradations. Chemical purity of the hormone following P-100 and CMC chromatography was evaluated using the phenyl isothiocyanate procedure of Edman (1960). Most degradations were carried out by the automated procedure (Edman and Begg, 1967); aliquots containing 3-5 nmol of peptide were subjected to several steps of degradation in the Beckman Sequencer, Model 201 (Beckman Instruments, Palo Alto, Calif.) using [^{35}S]phenyl isothiocyanate (Amersham-Searle, Arlington Heights, Ill.) to improve sensitivity of detection (Jacobs *et al.*, 1973). Manual degradations were

carried out using previously described techniques (Niall and Potts, 1970). Phenylthiohydantoin were identified by thin-layer chromatography accompanied by autoradiography (Edman and Begg, 1967; Jacobs *et al.*, 1973) and by gas-liquid chromatography (Pisano and Bronzert, 1969).

Amino Acid Analyses. Amino acid analysis was carried out both to provide compositional information and to measure protein content of the purified preparations for use in standardizing the radioimmunoassays and biological assays. All hydrolyses were performed using 5.7 N HCl containing 1:2000 (v/v) mercaptoethanol (Keutmann and Potts, 1969). Analyses were carried out using the Beckman Model 121 automatic amino acid analyzer. Amino acids were normalized from mole fractions into moles per mole by best fit based upon recovery of all stable residues.

Bioassays. The *in vitro* potency of the CMC-purified human hormone was assessed using the rat renal-cortical adenylyl cyclase assay system as described by Marcus and Aurbach (1969). [^{32}P]ATP and [^3H]cAMP were purchased from New England Nuclear and Schwarz/Mann, respectively. Medical Research Council (MRC) preparation 72/286 (National Institute for Medical Research, Mill Hill, London, England), a highly purified bovine hormone preparation, was used as reference standard.

Results

Preparation of CCl_3COOH -HPTH. Following drying and defatting, 96 g of acetone powder was obtained from 520 g of pooled tissue. The initial extraction of this preparation yielded 394 mg of CCl_3COOH precipitate (AT-1, Table I) containing 16 mg of hormone by radioimmunoassay.

Immunoassay of the acetone powder itself after small-scale extraction by either urea-acetic acid or phenol showed, however, an immunoreactive hormone content of 80-90 mg (AP, Table I).

Therefore, it was felt that additional quantities of hormone might be obtained by a series of reextractions of the residues from the initial extraction procedure. The tissue residue from the original phenol step was thus extracted and processed a second and third time (Figure 1), yielding an additional 5.5 mg of immunoreactive hormone (BT-1, CT-1; Table I). In addition to these reextractions of the phenol residue, the ether-powder residues from the first two extractions (AE, BE;

TABLE I: Recovery of Immunoassayable Hormone from Extraction of 520 g of Pooled Human Parathyroid Gland Tissue.

Preparation	Dry Weight	Hormone Content
Acetone powder (AP)	96 g	85 mg
CCl_3COOH precipitates		
AT-1	394 mg	16.0 mg
AT-2	160	2.0
BT-1	218	4.5
BT-2	38	0.3
CT-1	124	1.0

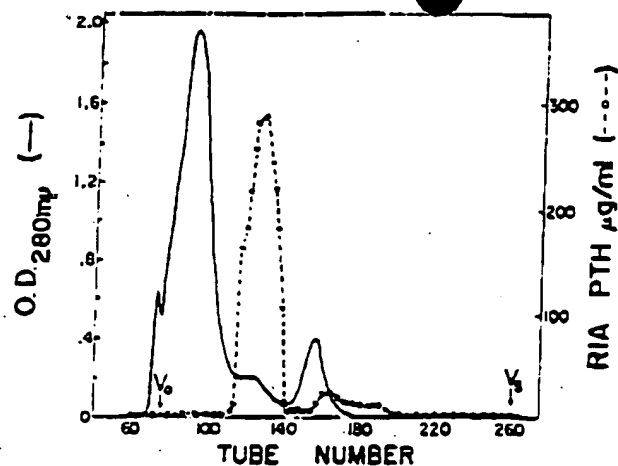


FIGURE 3: Elution profile obtained by passage of 273 mg of CCl_3COOH -HPTH (AT-1) through a 140×2.5 cm column of Bio-Gel P-100 using 0.14 M ammonium acetate buffer (pH 4.9). Fraction size was 2.2 ml. Parathyroid hormone, detected by radioimmunoassay, eluted between tubes 118 and 140.

Figure 1) were also carried through repeat salt treatment and CCl_3COOH precipitations. This resulted in a further, though modest, enhancement of recovery (AT-2, BT-2; Table I). Overall, 24 mg of immunoassayable parathyroid hormone was recovered at the CCl_3COOH stage; hormone content of the CCl_3COOH precipitates ranged from 4 (AT-1) to 1.5% (BT-2).

The slope of the displacement curves for the various preparations at the successive steps of extraction were identical with one another (Figure 2a) and with the HPTH standard (HT-67) prepared from the earlier extracts of O'Riordan *et al.* (1971b) (Figure 2b). Nevertheless, the CCl_3COOH preparations from the various extracts were kept separate as far as was practical through the subsequent stages of purification.

Column Purification. A series of column purifications was performed using successive steps of gel filtration and ion-exchange chromatography. For simplicity the main purification procedure (CCl_3COOH preparation AT-1) is described in detail, and this is followed by consideration of the pertinent features of the purification of the CCl_3COOH preparations derived from the various reextractions.

Purification of HPTH from CCl_3COOH Preparation AT-1. Figure 3 shows the elution profile obtained from P-100 gel filtration of 273 mg of CCl_3COOH preparation AT-1. The peak of immunological activity corresponded to a K_A of 0.3, an elution position similar to that observed for bovine parathyroid hormone using the same type of column, and somewhat earlier than that for BPTH using Sephadex G-100 (Kutmann *et al.*, 1971). An aliquot of the pooled peptide from this region (tubes 113-142) was analyzed by automated Edman degradation. Two predominant amino acids were found at each cycle, in essentially equivalent yields (Figure 4A): step 1, Ser, Val; step 2, Val, Leu; step 3, Ser, His; step 4, Glu, Pro; step 5, Ile, Ala.

The pooled P-100 preparation was subjected to ion-exchange chromatography on carboxymethylcellulose; the elution profile is shown in Figure 5. All of the immunoreactive hormone eluted in a single peak at a conductivity of 7-9 mmhos, a position comparable to that observed for chromatography of BPTH in the same system. Aliquots were taken from across the peak (tubes 124, 128, and 130; Figure 5), as well as from selected tubes adjacent to the peak, for automated Edman degradation. The peptide from all tubes within the

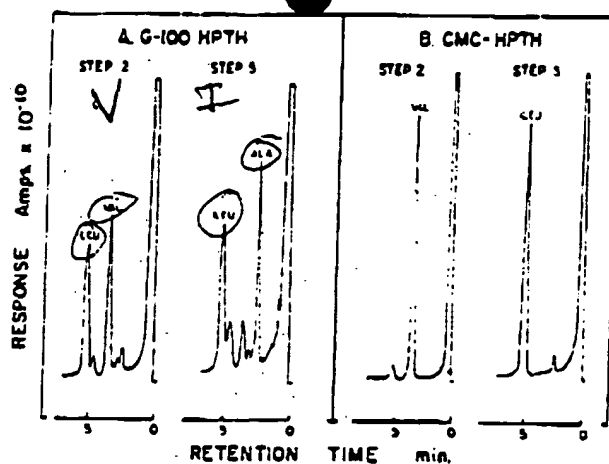


FIGURE 4: Gas-liquid chromatography of phenylthiohydantoin from selected steps of automated Edman degradation of human parathyroid hormone at successive stages of column purification. Valine and isoleucine was derived from the HPTH; the leucine and alanine represented a nonhormonal contaminant present in the P-100 preparation (A) which was eliminated by CMC chromatography (B). The identifications were carried out using a DC-560 column at 180° ; isoleucine was confirmed by repeat injection onto an AN-600 column.

peak retained the sequence Ser-Val-Ser-Glu-Ile-, while a peptide with the sequence Val-Leu-His-Pro-Ala- was found in the eluate fractions following the peak of immunoreactive hormone. This peptide, devoid of immunoreactivity, was felt to represent a non-hormonal contaminant which was responsible for the double sequence seen in Edman degradation of the earlier P-100 pool.

The hormone fractions were pooled and lyophilized. Purity of the peptide (CMC-HPTH) in this pool was found to be 95% based on multiple steps of Edman degradation (Figure 4B). Identical profiles and results were obtained from P-100 and CMC purification of a second lot (107 mg) of CCl_3COOH preparation AT-1.

Purification of HPTH from Reextract CCl_3COOH Precipitates. A third and fourth Bio-Gel P-100 column run was carried out with the reextract CCl_3COOH precipitates, and the elution profiles were closely similar to those described for the preparation AT-1. However, the purity of the hormone in the respective pooled eluates, again assessed by automated Edman degradation, was found to be lower. In the third P-100 pool, derived principally from preparations BT-1 and

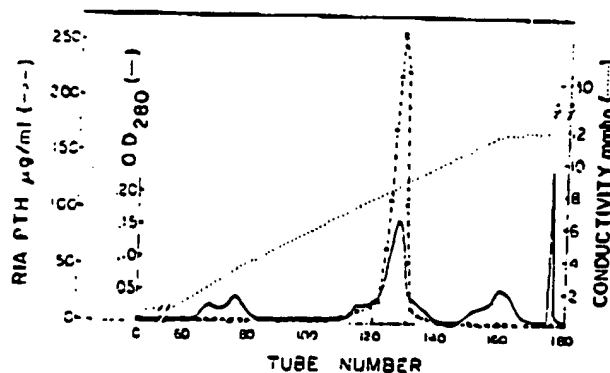


FIGURE 5: Elution profile from chromatography of a P-100 pool of HPTH (Figure 3) on a 10×0.9 cm column of carboxymethylcellulose using an ammonium acetate buffer gradient as described in text. Fraction size was 1.8 ml. Hormone eluted between tubes 121 and 131.

TABLE II: *In Vitro* Renal Adenylyl Cyclase Activity of Purified Parathyroid Hormone from Various Species.

Hormone	Act. (MRC Units/mg) ^a
Bovine I	3000 (2500-4000)
Porcine	1000 (850-1250)
Human	350 (275-425)

^a Activity expressed as mean potency with 95% confidence limits.

BT-2. HPTH represented about 25% of the total peptide while in the fourth pool, comprised principally of preparation CT-1, the per cent HPTH was even lower.

CMC chromatography on a pilot scale of material from one of these reextract pools showed that, while the pattern of eluted immunoreactivity remained the same as that for extract AT-1 (Figure 5), the purity of pooled hormone was, as with the pools from gel filtration, significantly lower. In particular, the principal non-hormonal peptide (Val-Leu-His-Pro-Ala-) was found to overlap the later fractions of the immunoreactive hormone peak.

Therefore, the further purification of the reextract P-100 preparations was modified to include a step of CMC chromatography in 8 M urea. Several additional peaks of optical density were found across the profile, but the immunoreactive hormone again eluted in a single peak, at a conductivity of 4.5 mNhos. The eluate pool from this region was diluted with distilled water (to lower the ionic strength) and reappplied to CMC in order to remove the urea. When a gradient was developed using urea-free buffer, the hormone eluted in a profile again resembling that shown in Figure 5. The hormone in this product was 85-90% pure by end-group analysis. A final passage through CMC, using the same urea-free buffer system, increased the purity to a level comparable to that of the CMC-HPTH from the original AT-1 preparation.

Recovery Estimates. In the purification of the initial CCl₃COOH preparation AT-1, recovery of immunoassayable hormone was found to be similar at each step: 62% from Bio-Gel P-100 and 56% from CMC. Recoveries from P-100 gel filtration of the two reextract CCl₃COOH preparations were somewhat lower: 41 and 45%, respectively. Per cent recovery of these preparations from CMC chromatography was closely similar to the AT-1 material. It was found that use of urea-CMC chromatography was not accompanied by any significant enhancement in recovery. The total yield of highly purified CMC-HPTH, suitable for structural studies, from purification of all the CCl₃COOH preparations was 3.2 mg as calculated from the amino acid analyses.

Biological Assays. Three preparations of CMC-HPTH, quantitated by amino acid analysis, were tested in the renal-cortical adenylyl cyclase system. The assays were found to be statistically homogeneous with a mean potency of 350 MRC units/mg. The relative potencies of human, bovine, and porcine parathyroid hormone in this assay system are compared in Table II.

Amino Acid Composition. The compositional analysis of CMC HPTH, based on duplicate hydrolysis for 24 and 72 hr, is outlined in Table III. The compositions of BPTH (Keutmann *et al.*, 1971) and PPTH (Woodhead *et al.*, 1971) are also shown for comparison. Results of additional 24-hr hydrolyses of CMC-HPTH, carried out with other preparations for calibration of peptide weight, indicated that all the CMC

products (whether derived from AT-1 or from the various re-extracts) had the same amino acid compositions.

Discussion

Availability of parathyroid tissue starting material has been the limiting factor in efforts to purify human parathyroid hormone. It was also evident from the earlier experience of O'Riordan *et al.* (1971b) that additional problems could also be anticipated in successfully carrying out the purification of sufficient hormone for structural analysis. In particular, it was found that (a) yields of hormone from column chromatography, particularly Sephadex gel filtration, were low and (b) purity, by radioimmunoassay, of HPTH after CCl₃COOH precipitation and gel filtration was considerably lower than that found at the corresponding stages for bovine hormone.

Accordingly, in the current work modifications were made in the later stages of the purification procedure in an effort to offset these problems. Gel filtration was carried out on polyacrylamide instead of polydextran, since in our experience recoveries from the polyacrylamide are somewhat better, perhaps owing to less adsorption of peptide to the column packing. All columns were presaturated with bovine parathyroid hormone prior to use for the human hormone.

By use of these combined maneuvers, it would appear that considerable improvement in yield, especially at the gel filtration step, was obtained, although recoveries were still lower than those found in the course of purification of larger amounts of bovine hormone (Keutmann *et al.*, 1971). Purity of the hormone after gel filtration, as assessed by end-group analysis, was also greater than the estimate of 10% based on radioimmunoassay, reported previously (O'Riordan *et al.*, 1971b).

Use of the manual Edman end-group procedure (Edman, 1960) in monitoring chemical purity of hormone at successive isolation steps proved to be of great value in our earlier work on a larger scale with BPTH (Keutmann *et al.*, 1971). In the current work, with more limited amounts of material, the automated degradation was effectively employed as a rapid, efficient means of screening aliquots across peaks as well as pooled peptide. Only 3-5 nmol of peptide was required for a degradation of several steps, permitting an accurate computation of the content of hormone vs. contaminating peptides. Direct chemical evidence of peptide purity was thereby obtained, eliminating the need to consume material through other types of purity assessment such as electrophoretic or chromatographic procedures. The Edman degradation also was useful in providing assurance that the use of BPTH for presaturation of columns to minimize losses was a safe procedure, since in initial pilot-scale column runs the purified HPTH fractions were found to be free from alanine, the amino-terminal residue of BPTH.

The automated Edman degradations showed that a principal contaminant, devoid of immunoassayable parathyroid hormone activity, had been eliminated by the final step of purification (Figure 4). This contaminant appeared different by amino-terminal sequence from the two contaminating peptides which were encountered in the purification of BPTH (Keutmann *et al.*, 1971). Complete purification of the bovine hormone was accomplished by use of CMC chromatography with buffers containing urea. With the principal P-100 preparations of the human hormone, CMC chromatography in the absence of urea was sufficient to yield a pure product. This may be in part a reflection of the differences in contaminating peptides. Moreover, evidence for isohormonal forms,

TABLE III: Amino Acid Composition of Human Parathyroid Hormone.

Amino Acid	Residues Found (Mol Fraction)		Residues Taken ^a			
	24 hr	72 hr	Mol/Mol of Peptide	Mol Integer	BPTH I	PPTH
Aspartic acid	0.119	0.123	9.80	10	9	8
Threonine ^b	0.017	0.017	1.40	1	0	0
Serine ^b	0.074	0.075	6.10	6	8	8
Glutamic acid	0.118	0.113	9.70	10	11	11
Proline	0.027	0.032	2.30	2	2	2
Glycine	0.056	0.057	4.65	5	4	5
Alanine	0.090	0.092	7.35	7	7	6
Valine	0.093	0.094	7.60	8	8	9
Methionine	0.022	0.015	1.80	2	2	1
Isoleucine	0.012	0.011	0.95	1	3	3
Leucine	0.119	0.116	9.80	10	8	10
Tyrosine	0.002	0.002	0.15	0	1	0
Phenylalanine	0.021	0.020	1.65	2	2	1
Lysine	0.113	0.112	9.30	9	9	9
Histidine	0.058	0.062	4.75	5	4	5
Arginine	0.059	0.059	4.80	5	5	5

^a The limited quantities of purified peptide available precluded more detailed studies of amino acid composition based on total enzymatic hydrolysis and acid hydrolysis for 24, 48, 72, and 96 hr, to determine the extent of amidation of aspartic and glutamic acid and content of tryptophan, and to more closely examine the content of certain low-yield residues (such as threonine) found to differ in amount from integral values of moles of residue per mole of peptide. ^b Calculated after extrapolation to zero hydrolysis time.

such as those found after urea-CMC chromatography of BPTH (Keutmann *et al.*, 1971), was not found in any of the eluates of the human hormone.

Ion-exchange chromatography in the presence of urea was, however, a useful step in processing P-100 preparations of lower purity, namely, those originating from the tissue re-extractions (Figure 1). Keeping the CCl₃COOH preparations from the initial extraction and from the re-extracts separate for subsequent purification is therefore clearly warranted. The final purified products from all of the respective extractions were, however, identical by radioimmunoassay and composition.

While the improved yields from the chromatographic steps were gratifying, assessment of yields of hormone from the earlier extraction stages (Table I) showed that substantial quantities of immunoreactive hormone found in the original acetone powder were not accounted for at the CCl₃COOH stage. Hence, the 394 mg of CCl₃COOH precipitate (AT-1) from the initial extraction contained 16 mg of hormone, compared with 85 mg in the acetone powder starting material. For this reason, the various re-extractions of acetone powder residue and ether powder were carried out. These yielded additional amounts of hormone, but the total recovery at the CCl₃COOH stage was still only 30%.

The basis for this apparent loss remains uncertain. The greatest drop in immunoreactive hormone content appeared to occur between the acetone powder and ether powder stages. Little or no loss took place at the salt precipitation stage, but appreciable loss again occurred at the CCl₃COOH step. Relevant to this is the finding that re-extraction of the original phenol residue yielded more hormone than did re-extraction of the ether powder (Table I).

Conceivably, some of the immunoreactivity in the acetone powder could represent fragments which are separated out along the way, despite the fact that the displacement curves

for the hormone from the successive stages of extraction appeared to be parallel.

Prior to their independent sequence analysis of the active amino-terminal region of the molecule, Brewer *et al.* (1972) purified the human hormone starting with a similar quantity of pooled parathyroid tissue. Their procedure employed chloroform, instead of acetone, for drying and defatting of the tissue, and urea-hydrochloric acid rather than phenol for extraction (Arnaud *et al.*, 1970). Although a detailed description of their purification is as yet unavailable, their overall recovery appears to have been similar to our own.

Substitution of serine for alanine at the amino terminus may contribute to the low activity (350 MRC units/mg) of native HPTH, compared to that of the bovine hormone, when assessed by the *in vitro* renal adenyl cyclase assay. Earlier studies had demonstrated that porcine parathyroid hormone (PPTH), which also contains an amino-terminal serine residue, likewise shows a lower activity relative to BPTH in the same assay system (Woodhead *et al.*, 1971).

The compositional data obtained in these studies (Table III) point toward significant structural features to be anticipated in analysis of the carboxyl-terminal region of the molecule. Certain similarities to porcine parathyroid hormone may be discerned, including a similar distribution of basic residues, high content of leucine, and absence of tyrosine. However, several differences from both the porcine and bovine molecules are evident as well.

Although the structure for the amino-terminal region proposed by ourselves (Niall *et al.*, 1974) differs in certain respects from that reported by Brewer *et al.* (1972), it is clear from the compositional studies that, regardless of how these discrepancies are resolved, several amino acids unique to the composition of HPTH should be found in the sequence of the carboxyl-terminal portion. For example, threonine and an aspartic acid residue remain to be located. Also, since the single

isoleucine has already been located (position 5), substitutions would be expected at the two positions occupied by residue in the carboxyl terminus of BPTH and PPTH. Presence in the carboxyl-terminal region of several sequence positions unique to HPTH could account for the low cross-reactivity observed with HPTH against a number of carboxyl-terminal directed anti-bovine antisera (Segre *et al.*, 1972).

The purification of human parathyroid hormone has not only made available peptide for structural analysis, but also has provided a standard, calibrated against multiple CMC preparations, which should be very suitable for future immunological studies. The correlations of structure, biological activity and immunoreactivity which should now be possible will represent important advances toward defining the significance of the various forms of the hormone found in the human circulation.

Acknowledgments

We acknowledge with gratitude the large number of internists, surgeons, and pathologists from medical centers throughout the United Kingdom, United States, and western Europe whose generous contributions of human parathyroid tissue made this investigation possible. We express our appreciation to Mrs. H. Pilling, Medical Unit, Middlesex Hospital for her invaluable assistance in coordinating the collection of parathyroid tissue. We also acknowledge the expert technical assistance of Mr. Edward Callahan, Mr. John Jacobs, Miss Barbara Miller, and Miss Karen Swenson.

ADDED IN PROOF

Subsequent evaluation of the supernatant fraction from CCl₃COOH precipitation (AT-1, Figure 1) showed that a small quantity of intact hormone (about 10% of that appearing in the precipitate) remained behind after the precipitation. This hormone was retrieved in good yield for further purification by the following procedure. The supernatant was extracted with ether to remove trichloroacetic acid. The content of acetic acid (lowered by the ether extraction) was restored to approximately 20%. The hormone was then extracted into 1-butanol, and the butanol removed by rotary evaporation.

References

- Addison, G. M., Hales, C. N., Woodhead, J. S., and O'Riordan, J. L. H. (1971), *J. Endocrinol.* 49, 521.
- Arnaud, C. D., Tsao, H. S., and Oldham, S. B. (1970), *Proc. Nat. Acad. Sci. U. S.* 67, 415.
- Aurbach, G. D. (1959), *J. Biol. Chem.* 234, 3179.
- Berson, S. A., Yalow, R. S., Aurbach, G. D., and Potts, J. T., Jr. (1963), *Proc. Nat. Acad. Sci. U. S.* 49, 613.
- Brewer, H. B., Jr., Fairwell, T., Ronan, R., Sizemore, G. W., and Arnaud, C. D. (1972), *Proc. Nat. Acad. Sci. U. S.* 69, 3585.
- Brewer, H. B., Jr., and Ronan, R. (1970), *Proc. Nat. Acad. Sci. U. S.* 67, 1862.
- Canterbury, J. M., and Reiss, E. (1972), *Proc. Soc. Exp. Biol. Med.* 140, 1393.
- Cohn, D. V., MacGregor, R. R., Chu, L. L. M., Kimmel, J. R., and Hamilton, J. W. (1972), *Proc. Nat. Acad. Sci. U. S.* 69, 643.
- Edman, P. (1960), *Ann. N. Y. Acad. Sci.* 88, 602.
- Edman, P., and Begg, G. (1967), *Eur. J. Biochem.* 1, 80.
- Goldsmith, R. S., Furszyfer, J., Johnson, W. J., Fournier, A. E., Sizemore, G. W., and Arnaud, C. D. (1973), *J. Clin. Invest.* 52, 173.
- Habener, J. F., Kemper, B., Potts, J. T., Jr., and Rich, A. (1972), *Science* 178, 630.
- Habener, J. F., Powell, D., Murray, T. M., Mayer, G. P., and Potts, J. T., Jr. (1971), *Proc. Nat. Acad. Sci. U. S.* 68, 2986.
- Jacobs, J. W., Sauer, R. T., Niall, H. D., Keutmann, H. T., Aurbach, G. D., and Potts, J. T., Jr. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 32, 2445.
- Keutmann, H. T., Aurbach, G. D., Dawson, B. F., Niall, H. D., Duftos, L. J., and Potts, J. T., Jr. (1971), *Biochemistry* 10, 2779.
- Keutmann, H. T., and Potts, J. T., Jr. (1969), *Anal. Biochem.* 29, 175.
- Marcus, R., and Aurbach, G. D. (1969), *Endocrinology* 85, 801.
- Niall, H. D., Keutmann, H., Sauer, R., Hogan, M., Dawson, B., Aurbach, G., and Potts, J. T., Jr. (1970), *Hupke-Seyler's Z. Physiol. Chem.* 351, 1586.
- Niall, H. D., and Potts, J. T., Jr. (1970), in *Peptides: Chemistry and Biochemistry, Proceedings of the First Biochemistry Symposium*, New York, N. Y., Marcel Dekker, p 215.
- Niall, H. D., Sauer, R. T., Jacobs, J. W., Keutmann, H. T., Segre, G. V., O'Riordan, J. L. H., Aurbach, G. D., and Potts, J. T., Jr. (1974), *Proc. Nat. Acad. Sci. U. S.* 71, 384.
- O'Riordan, J. L. H., Aurbach, G. D., and Potts, J. T., Jr. (1969), *Proc. Nat. Acad. Sci.* 63, 692.
- O'Riordan, J. L. H., Potts, J. T., Jr., and Aurbach, G. D. (1971b), *Endocrinology* 89, 234.
- O'Riordan, J. L. H., Woodhead, J. S., Addison, G. M., Keutmann, H. T., and Potts, J. T., Jr. (1972), in *Endocrinology 1971: Proceedings of the Third International Symposium*, Taylor, S., Ed., London, Heinemann Books Ltd., p 386.
- O'Riordan, J. L. H., Woodhead, J. S., Robinson, C. J., Parsons, J. A., Keutmann, H., Niall, H., and Potts, J. T., Jr. (1971a), *Proc. Roy. Soc. Med.* 64, 1263.
- Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem.* 244, 5597.
- Segre, G. V., Habener, J. F., Powell, D., Tregear, G. W., and Potts, J. T., Jr. (1972), *J. Clin. Invest.* 51, 3163.
- Silverman, R., and Yalow, R. S. (1973), *J. Clin. Invest.* 52, 1958.
- Woodhead, J. S., O'Riordan, J. L. H., Keutmann, H. T., Stoltz, M. L., Dawson, B. F., Niall, H. D., Robinson, C. J., and Potts, J. T., Jr. (1971), *Biochemistry* 10, 2787.

cells contain a molecule similar to the i-ag's. An antiserum produced against these extracts agglutinates both axenic and nonaxenic cells. Fluorescence in immunolabeled cells is principally associated with buccal structures. Labeling of surface extracts blotted on nitrocellulose or DBM paper reveals 2 main antigens. One of them is present on both types of cells, while the other, i-ag like, is only found in nonaxenic cells. Neither of the 2 components is detected in exponentially growing cells. Results suggest that i-ag of ciliates, whose function is at present unknown, could play a role in feeding processes.

Reduction of the number of oligodendrocytes enhances their proliferation rate

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We have previously shown that the proliferation rate of oligodendrocytes (O) in culture is age-dependent. We have now investigated whether the genetically determined proliferation rate of O can be influenced by extrinsic factors. In neonatal mouse brain cell cultures, O carrying the antigenic marker galactocerebroside (GC) were eliminated by complement dependent anti-GC antibody mediated cytotoxicity at day 14 in vitro. 4 days after the withdrawal of the cytotoxic medium, 20% of the normal number of GC⁺O reappeared. Their proliferation rate at that time was estimated by ³H-thymidine autoradiography combined with GC immunostaining. The proliferation rate of O in 18-day-old untreated cultures was 13±1.9%. The proliferation rate of O in cultures where their number was reduced by cytotoxicity was enhanced to 26.6±5.1%. These results indicate that reduction of the number of O in culture increased the proliferation rate of O.

Independent lateral mobility of the (Na⁺, K⁺)-ATPase subunits in the plasma membrane of toad leukocytes

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The surface expression and lateral mobility of the glycoprotein and catalytic subunit of the (Na⁺, K⁺)-ATPase was studied on toad leukocytes by immunofluorescence and cytofluorometry using the biotin-streptavidin bridge technique. On fixed cells the distribution was homogeneous with complete overlapping of the 2 labeling patterns. On viable cells labeled at 4°C, patches were observed with little overlapping between the 2 subunits. After warming, each of the 2 subunits capped in a different area of the cell surface. When 1 subunit was capped and the cells fixed, the distribution of the other subunit was homogeneous. The kinetics of capping and reexpression of subunits at the cell surface were analyzed by cytofluorometry. Our results indicate that the 2 (Na⁺, K⁺)-ATPase subunits are loosely linked in the plasma membrane.

Expression and processing of human preproparathyroid hormone in *Escherichia coli*

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In order to study processing and secretion of human preproparathyroid hormone (hpreproPTH) in *E.coli*, we

have constructed vectors designed to efficiently express the previously cloned complementary DNA (cDNA) for hpreproPTH in transformed bacteria using a previously described approach. A *lac* promoter DNA fragment including a ribosomal-binding site was placed at varying distances in front of the coding sequence for a hybrid protein fusing the 'prepro' coding sequence of hpreproPTH and the enzymatically active carboxyterminal fragment of β -galactosidase. Clones expressing hybrid protein were recognized in indicator agar plates. Using plasmids from clones efficiently expressing the hybrid protein, the entire coding sequence for hpreproPTH was reconstructed. Plasmid encoded proteins were radiolabeled using the maxicell technique. On gel electrophoresis 2 immunoprecipitable peptides have been identified. The larger has the size of hpreproPTH, and the smaller comigrates with hPTH, suggesting that the hpreproPTH is correctly processed. After cell fractionation β -lactamase, but not hPTH, is found soluble in the periplasmic space.

The affinities of myomal and myometrial progesterin receptors to different steroids

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Benign tumors of the smooth muscle are rare in human beings except in the uterus. Here they are frequent but grow only in presence of active ovaries indicating that tumorous and nontumorous cells respond differently to identical ovarian stimuli. In the present work we determined the affinities of various steroids to the progesterin receptors present in the 100,000×g supernatant of the 2 tissues. This was accomplished with a competitive binding assay using promegestone as labeled ligand. The 2 receptors had the same affinities to all steroids as long as they had the same conformation as progesterone in either the A or D ring. However, if the conformation of both was altered as in 17 α -hydroxypregnenolone, 20 α -hydroxypregnenolone and dehydroepiandrosterone then the receptors of the tumor exhibited a higher affinity. Thus, subtle differences exist between the steroid binding sites of the 2 receptors. This could in part be responsible for the different responses of the 2 tissues to identical ovarian stimuli.

Glucocorticoid regulation by mouse mammary tumor virus (MMTV) DNA

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To characterize the regulatory region of viral DNA, we have joined a cloned MMTV DNA fragment containing the long terminal repeat (LTR) to a DNA fragment containing the coding sequence of the herpes simplex thymidine kinase gene lacking its own promoter. Introduction of this hybrid molecule into mouse Ltk⁻ cells by transfection yielded tk-positive cell clones. Deletion mutants lacking portions of the LTR upstream of the viral promoter were constructed and introduced into Ltk⁻ cells. Tk⁺ clones were obtained, and their pattern of transcription will be reported.

Effect of DNA methylation on globin gene expression

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Using a novel DNA methylation technique, we set out to identify DNA sequences which are responsible for the effect of DNA methylation on gene expression. The method used allows to in vitro-methylate specific segments of cloned eukaryotic DNA, which is then brought back into

SOLUTION SYNTHESIS OF [ASN⁷⁶]-HUMAN PARATHYROID HORMONE(1-84)

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Human parathyroid hormone, hPTH(1-84), was synthesized by the conventional solution procedure applying the maximal-protection approach. All protecting groups were removed simultaneously by the HF method. The product was purified by CM-cellulose column chromatography, gel-filtration on Sephadex G-50 and in the final stage, by reversed phase HPLC. The structure of the final product was confirmed not only by HPLC analysis but also by peptide mapping of tryptic digests on HPLC. The present product showed 350(249-480) IU/mg on in vitro rat renal adenylate cyclase assay.

The amino acid sequence of human parathyroid hormone, hPTH, was originally determined by Keutmann *et al.* (1) in 1978 as a single-chain polypeptide with 84 amino acid residues, and the total synthesis was successfully done for the first time by our research group in 1981 (2). Immediately after our synthesis, the structure was revised by Hendy *et al.* (3) to have Asn instead of Asp at position 76 in Keutmann's structure, based on deduction from sequence analysis of cDNA cloned for human preproPTH. This communication reports the solution synthesis of [Asn⁷⁶]-hPTH(1-84) and the characterization of the product.

MATERIALS AND METHODS

Materials. DPCC-treated trypsin(EC 3.4.21.4.) was purchased from Sigma Chemicals Co., and amino peptidase-M(EC 3.4.11.2.) was purchased from Pierce Chemical Co. Boc-amino acids and other reagents for peptide synthesis were obtained from Peptide Institute, Inc., Osaka, Japan.

Abbreviations: WSCI, water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HOBT, 1-hydroxybenzotriazole; Pac, phenacyl ester; DMF, N,N-dimethylformamide; DPCC, diphenyl carbamyl chloride.

Peptide Synthesis. [Asn⁷⁶]-hPTH was synthesized by the conventional solution procedure as shown in Fig. 1. Condensation reaction of each segment was carried out in DMF or N-methylpyrrolidone by the WSCI/HOBT method after removal of the terminal Boc- or Pac group. The fully protected product was deprotected by the HF method using an HF reaction apparatus, Protein Research Foundation Type I. The crude product was purified on a column of CM-cellulose, Sephadex G-50 and then by reversed-phase HPLC.

Reversed-Phase HPLC. HPLC was performed on a Hitachi Liquid Chromatograph Model 638 equipped with a column of Nucleosil 5C₁₈ (150 x 4 mm). All runs were performed at ambient temperature at a flow rate of 1.0 ml/min. Other conditions are given in each figure legend.

HPLC Mapping of Tryptic Digests. A solution of Peptide (40 µg) in 40 µl of water were treated with a water-solution of DPCC treated trypsin (2.5 µg/5 µl) at 37°C; pH of the mixture was 6. After 30 min; 12 µl of the whole mixture was applied to the reversed-phase HPLC system.

Biological activity. Biological potency of hPTH was measured by *in vitro* assay of the rat-kidney adenylate cyclase activity following the procedure developed by Marcus and Aurbach (4) using WHO bovine PTH(1-84) as the standard.

RESULTS AND DISCUSSION

The principle of the present synthesis is based on the maximum protection strategy using stable protective groups at the side chains (5). The whole molecule was assembled with 13 segments by the route shown in Fig. 1. Boc-amino acids developed for Merrifield's solid phase procedure were used for stepwise synthesis of each segment in solution, which was started from the C-terminal amino acid Pac ester by the WSCI/HOBT method or by the active ester method. Each segment was also coupled by the WSCI/HOBT method after removal of the terminal Boc or Pac group; the latter group was removed by warming the peptide with Zn powder in acetic acid. No particular difficulty was encountered in the segment condensation reactions in solution when DMF or N-methyl-pyrrolidone was used as the solvent.

The fully protected 84-peptide thus obtained was treated with HF at 0°C for 1 hr in the presence of anisole, methionine, dimethylsulfide and ethanedithiol as scavengers. The crude

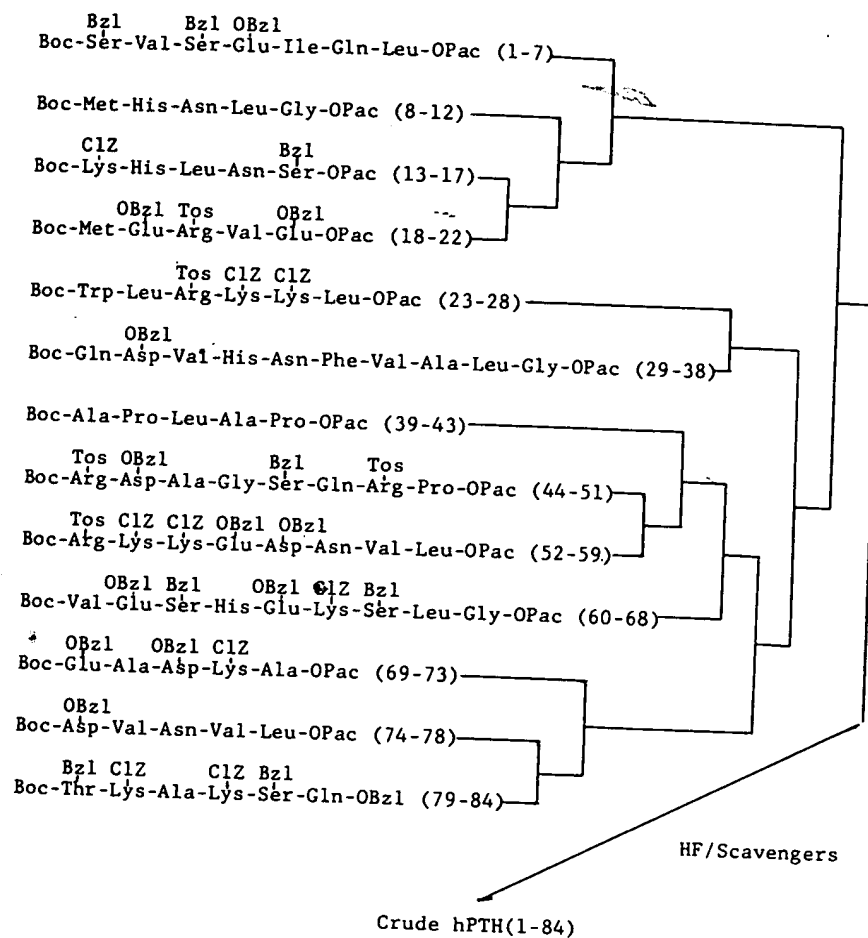


Fig. 1. Coupling route for the synthesis of [Asn⁷⁶]-hPTH(1-84).

product was fractionated on a CM-cellulose column using ammonium acetate solution with linear gradient concentrations from 0.05 M at pH 5 to 0.4 M at pH 6, and then by gel-filtration on Sephadex G-50 using 1 M acetic acid as the solvent. The main peak of the Sephadex chromatogram was developed on a gradient HPLC system (Fig. 2); the main peak here was collected from several different runs and rechromatographed on an isocratic HPLC system; the elution profile is shown in Fig. 3. The homogeneity of each fraction of the broad peak was checked with a similar HPLC system under analytical conditions; fractions showing a single peak on

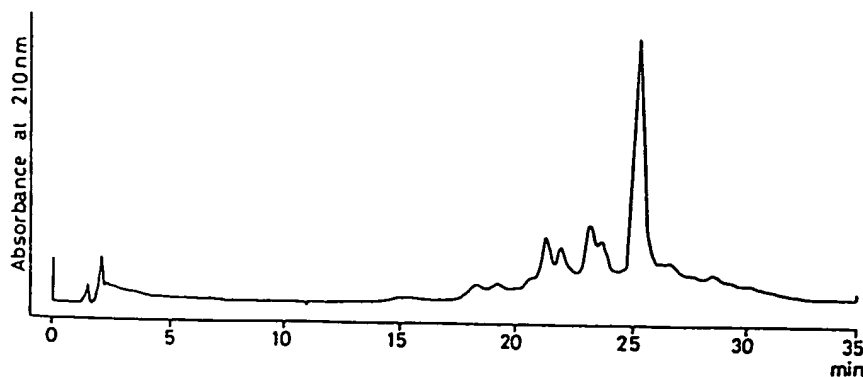


Fig. 2. HPLC profile of crude product obtained after gel-filtration on Sephadex G-50.
Eluant: 0.1 M NaCl(pH 2.4) containing MeCN, which was gradually increased from 27.5% to 40%.

analytical HPLC were collected and lyophilized to obtain a final product.

Amino acid analysis of an acid hydrolyzate and an Ap-M digest revealed that the product contained all component amino acids in the expected ratios (Table 1). When the final product was treated with a dilute aqueous hydrogen peroxide solution, the reaction mixture showed four peaks on HPLC including that of the intact molecule (Fig. 4). The formation of three additional

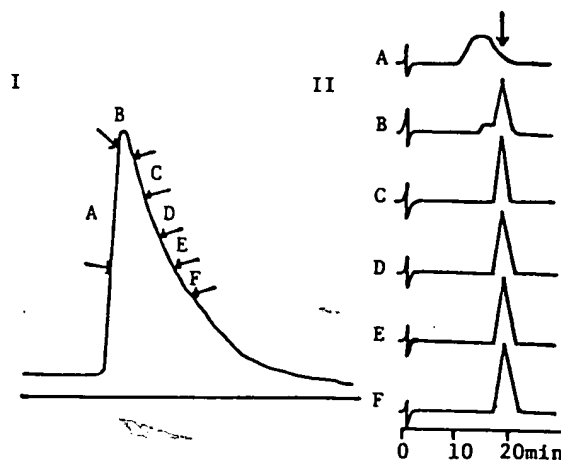


Fig. 3. Separation of final product, h-PTH(1-84), on HPLC.
I. Isocratic HPLC profile of collected materials.
II. Purity check of each fraction in I under analytical conditions; fractions C,D,E and F were pooled for lyophilization as the purified material. Eluant: 31% MeCN in 0.1 M NaCl(pH 2.4).

Table 1. Amino acid analyses of synthetic [Asn⁷⁶]-hPTH.

Amino Acid	Expected	6 N-HCl	Ap-M
Lys	9	9.36	8.92
His	4	3.60	3.60
NH ₂	9	10.08	
Arg	5	5.15	
Asp	5		3.76
Asp+Asn	10	10.00	
Thr	1	0.99	
Thr+Gln	5		3.85
Ser	7	5.74	
Ser+Asn	12		11.40
Glu+Gln	11	10.32	
Cit+Glu	12		10.80
Pro	3	2.74	3.24
Gly	4	4.00	4.00
Ala	7	7.07	6.86
Val	8	7.76	7.52
Met	2	1.20	1.12
Ile	1	0.77	0.90
Leu	10	9.90	9.20
Phe	1	1.01	1.04
Trp	1	0.53	0.52

peaks may be explained by the partial or complete oxidation of the Met residues to the sulfoxides since the hPTH molecule contains two Met residues at positions 8 and 18. This observation proved that the purified material was free of contamination from oxidized peptides.

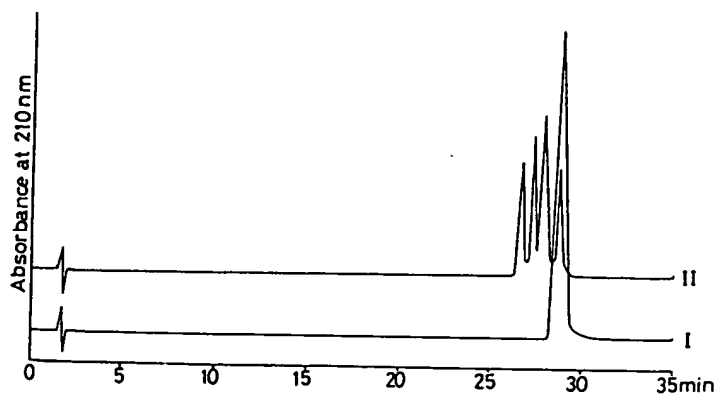


Fig. 4. HPLC profile of purified synthetic hPTH(1-84) and its hydrogen peroxide-treated products. I. hPTH(1-84) II. Oxidized products. Eluant: 0.1 M NaCl (pH 2.4) containing MeCN, which was gradiently increased from 10% to 50%.

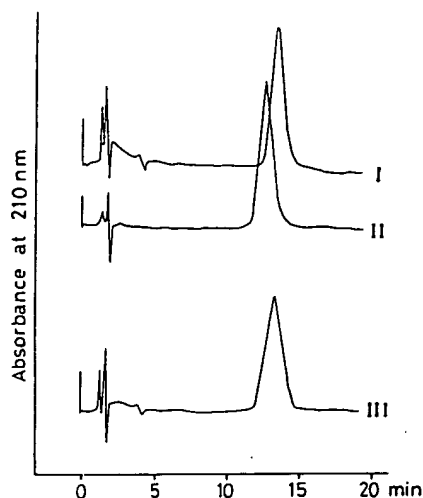


Fig. 5. Separation of Asn- and Asp-hPTH(1-84) on HPLC.

I. [Asp⁷⁶]-hPTH II. [Asn⁷⁶]-hPTH III. Mixture of Asp- and Asn-PTH. Eluant: 31% MeCN in 0.1 M NaCl (pH 2.0).

Our HPLC system showed enough resolution power for analyzing analogs of hPTH(23-84) containing Asp or Asn at position 76. However, the system failed to separate [Asn⁷⁶]- and [Asp⁷⁶]-hPTH(1-84) molecules into two peaks when they were injected onto the column as a mixture (see Fig. 5). Thus, the homogeneity had to be checked by other means. HPLC mapping of the trypsin digest was compared with those of some other synthetic peptides as references (Fig. 6). All peaks in the chromatograms were assigned as shown in the figure. In the chromatogram of the present product, all the expected fragments were observed and no unwanted peaks appeared. The present product might have been contaminated by D-Glu²²-containing peptide since the last coupling reaction was performed between segments(1-22) and (23-84), but no D-Glu²²-containing fragment(21-25) was detected by tryptic mapping within the range of resolution. Thus, We concluded that our present product was reasonably homogeneous. The product showed 350(249-480) IU/mg in the rat-kidney adenylate cyclase assay. The specific optical rotation value was observed to be: $[\alpha]_{D}^{20} -89.2^{\circ}$ (c 0.2, 1% AcOH).

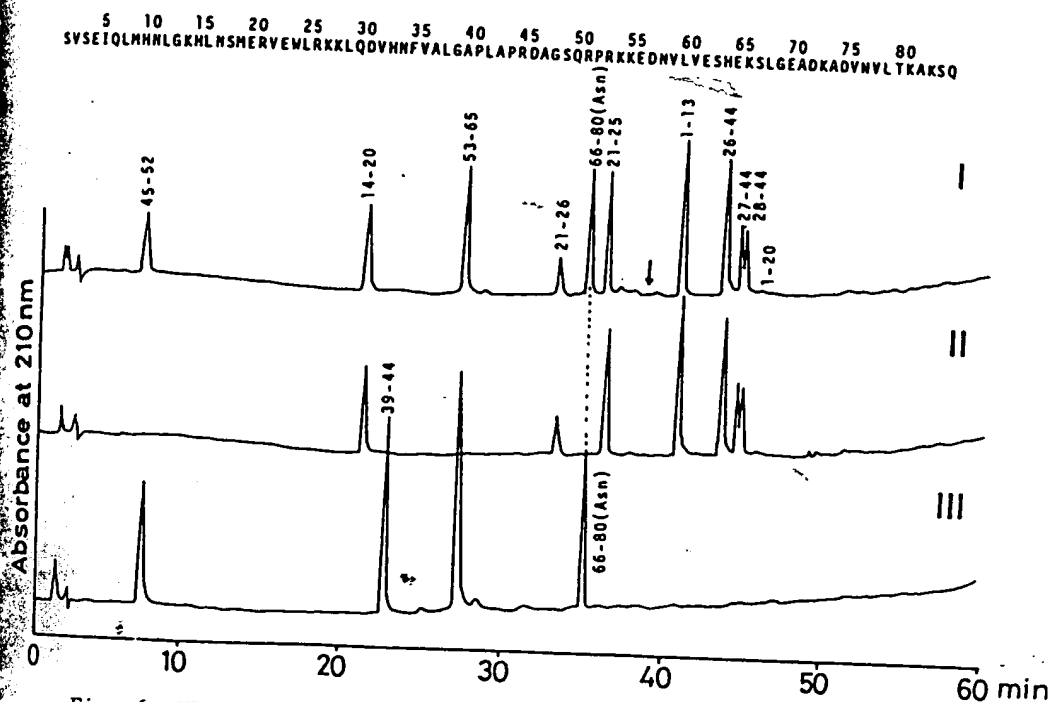


Fig. 6. HPLC mapping of a tryptic digest of [Asn⁷⁶]-hPTH in comparison with those of some other synthetic peptides. I. Asn-hPTH(1-84). II. hPTH(1-44) III. Asn-hPTH(39-84) Arrow indicates the position of [D-Glu²²]-containing fragment(21-25). Eluant: 10 mM phosphate buffer(pH 2.6) containing 50 mM Na₂SO₄ and MeCN, which was increased gradiently from 2% to 50%.

ACKNOWLEDGEMENT

We wish to thank Toyo Jozo Co., Ltd. for conducting the adenylate cyclase assay.

REFERENCES

1. Keutmann, H.T., Sauer, M.M., Hendy, G.H., O'Biordan, J.L.H., and Potts, Jr., J.T. (1978) *Biochemistry* 17, 5723-5729.
2. Kimura, T., Morikawa, T., Takai, M., and Sakakibara, S. (1981) *J. Chem. Soc. Chem. Commun.* 340-341.
3. Hendy, G.H., Kronenberg, H.M., Potts, Jr., J.T., and Rich, A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7365-7369.
4. Marcus, R. and Aurbach, G.D. (1969) *Endocrinology* 85, 801-810.
5. Kimura, T., Takai, M., Masui, Y., Morikawa, T., and Sakakibara, S. (1981) *Biopolymers* 20, 1823-1832.



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**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Paper No. 39

Application Number: 08/340,664
Filing Date: 11/16/94
Appellant(s): Gautvik et al.

Michele M. Schafer
For Appellant

5

EXAMINER'S ANSWER

10

This is in response to appellant's brief on appeal filed 5/26/99.

15

(1) *Real Party in Interest*

A statement identifying the real party in interest is contained in the brief.

(2) *Related Appeals and Interferences*

20

A statement identifying the related appeals and interferences which will directly affect or be directly affected by or have a bearing on the decision in the pending appeal (none) is contained in the brief.

(3) Status of Claims

The statement of the status of the claims contained in the brief is correct.

(4) Status of Amendments After Final

5 The appellant's statement of the status of amendments after final rejection contained in the brief is incorrect.

The amendment after final rejection filed on 5/31/99 has not been entered. That amendment was filed concurrently with the Appeal Brief, and raises new issues because of the recitation of 'recombinant' in the independent claim.

10

(5) Summary of Invention

The summary of invention contained in the brief is deficient because the information at page 4 of the Appeal Brief is not drawn to the disclosure, but to the comparison of recombinantly produced hPTH to synthetic hPTH as presented in declarations filed during prosecution. The summary of the invention as it appears at page 3 is correct.

15

(6) Issues

The appellant's statement of the issues in the brief is correct.

20

(7) Grouping of Claims

Appellant's brief includes a statement that claims 31-35 do not stand or fall together and provides reasons as set forth in 37 C.F.R. § 1.192(c)(7) and (c)(8).

(8) Claims Appealed

The copy of the appealed claims contained in Appendix 2 to the brief is correct.

(9) Prior Art of Record

The following is a listing of the prior art of record relied upon in the rejection of claims under appeal.

3,886,132

Brewer

5/27/95

E. Breyel et al., "Synthesis of Mature Human Parathyroid Hormone in Escherichia coli", Third European Congress on Biotechnology, Vol. III, 1984, p. 363-369.

W.L. Sung et al., "Hybrid gene synthesis: its application to the assembly of DNA sequences encoding the human parathyroid hormones and analogues", Biochem Cell Biol. 63:133-138, 1986.

K. Kaisha et al., GB 2 092 596 A, "Process for the production of human parathyroid hormone", 8/18/82.

H. Mayer, EP 0 139 076, 5/2/85.

(10) New Prior Art

No new prior art has been applied in this examiner's answer.

(11) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 33-35 are rejected under 35 U.S.C. 112, second paragraph, as being incomplete for omitting essential elements, such omission amounting to a gap between the elements. See MPEP § 2172.01. The omitted elements are: The specification discloses the invention to involve the expression of full-length hPTH(1-84) in yeast or *E. coli* by expressing a secretory peptide, e.g. hPTH fused to either the Staph. A signal sequence or the yeast Mat alpha signal sequence, such that the protein is secreted and processed by the host cell. The claims as they are currently written contain no reference to the secretory leader sequence, and recite only expression of hPTH(1-84), which is not described by the specification as originally filed. The omission of the sequence encoding the secretory leader amounts to a gap between the elements of the DNA to be expressed in the method recited in the claim, which is a product by process type claim.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. § 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless --

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

The following is a quotation of 35 U.S.C. § 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

5 Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

10 This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. § 103, the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 C.F.R. § 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of potential 35 U.S.C. § 102(f) or (g) prior art under 35 U.S.C. § 103.

15 Claims 31 and 32 are rejected under 35 U.S.C. § 102(b) as anticipated by or, in the alternative, under 35 U.S.C. § 103 as obvious over Brewer et al., U.S. Patent Number .

20 Brewer et al. disclose highly purified human PTH. See abstract, and col. 2 lines 49-60 wherein it is disclosed that the preparation was pure enough to sequence 34 amino acid residues starting at the amino terminus of the protein. Thus, the protein as purified by Brewer et al. appears to be consistent with the limitations of the instant claims with respect to being "substantially homogeneous" hPTH. It cannot be determined by the Examiner whether Brewer's protein specifically meets the limitations of being 95% pure, although it would seem likely that it did, given Brewer's ability to sequence 34 residues. It is noted that the only portion of the specification which relates to purity is the disclosure that the protein was partially sequenced (page 7, starting at line 27), which the ordinary artisan would recognize as requiring a relatively pure preparation of the desired protein (although no exact percentage purity can be implied). Based upon the fact that the specification discloses obtaining the sequence of 19 and 45 amino acids respectively, from the yeast and E. coli-produced protein, Brewer's ability to obtain 34 amino acids would seem to indicate that comparable purity was achieved. In the event Brewer's protein was less than 95% pure, it would have been obvious to further purify Brewer's protein using routine protein purification methodology, and one of ordinary skill in the art would have been motivated to do so in view of the known

pharmacological uses of the protein, and the art-recognized advantages of using the purest protein preparation possible for pharmaceutical use.

Claims 31-34 are rejected under 35 U.S.C. § 103 as being unpatentable over Breyel et al. (3rd Eur. Cong. Biotech., cited by appellants) or Sung et al. (Biochem Cell Biol. 64:133, cited by appellants) or Mayer et al. (EP 0 139 076, cited by appellants), any reference of the three in view of Kaisha et al. (GB 2 092 596, cited by appellants).

Breyel et al. teach expression of mature hPTH in *E. coli*, see Summary, page 363. The protein was expressed and bacterial cell extracts assayed for activity, see page 366 for example. Breyel differs from the instant claims only in that the protein was not purified from the bacterial cell extracts.

Sung et al. teach the construction of vectors for the direct expression of hPTH in bacterial, specifically *E. coli*, cells; see for example page 136, second column. At page 138, Sung et al. state "Study is now conducted in the expression of these gene products." Sung et al. do not actually disclose expression of the encoded protein or isolation of the expressed protein.

Mayer et al. teach recombinant production of hPTH in *E. coli*, see page 9, first full paragraph for example. The protein was purified from the cells and shown to be biologically active. Mayer et al. do not teach purification to the degree recited in the rejected claims.

Kaisha et al. teach a process for the production of hPTH. Although their patent is not drawn to recombinant production using bacterial or yeast cells, they disclose at page 2, first column, beginning at line 55 that:

"The hPTH thus obtained can be collected easily by purification and separation techniques using conventional procedures such as salting-out, dialysis, filtration, centrifugation, concentration and lyophilisation. If a more highly purified hPTH preparation is desirable, a preparation of the highest purity can be obtained by the above-mentioned techniques in combination with other conventional procedures such as adsorption and desorption with ion exchange, gel filtration, affinity chromatography, isoelectric point fractionation and electrophoresis."

Thus, Kaisha et al. teach the desirability of making large quantities of hPTH, and that the person of ordinary skill in the art, given a preparation containing hPTH, would be able to devise a protocol for purifying such with a reasonable expectation of success and without undue experimentation.

It would have been obvious to the person of ordinary skill in the art at the time the invention was made to express hPTH from the vector disclosed by Sung et al. or alternatively as taught by Breyel et al. and Mayer et al., and then to purify the hPTH so produced as suggested by Kaisha et al. to obtain highly purified hPTH. The ordinary artisan would have been motivated to do so in view of the art recognized desirability of obtaining hPTH in pure form, as evidenced by all three cited references. The teachings of Kaisha et al. indicate that the ordinary artisan would have had at least a reasonable expectation of success at purifying hPTH once produced as taught and/or suggested by Sung or Breyel or Mayer.

(12) New Ground of Rejection

This examiner's answer does not contain any new ground of rejection.

(13) Response to argument

With respect to the rejection under 35 U.S.C. §112, second paragraph, appellants argue at page 6 of the appeal brief that the use of a particular leader sequence is not the subject of the invention, and that the specification teaches that a variety of leader sequences may be used. This argument has been fully considered but is not deemed persuasive because it is appellants choice to define the product being claimed in a product-by-process format, and the particular manner in which the claim has been constructed omits what is disclosed as being an essential element, that is, that the protein must be expressed as a fusion protein comprising a leader sequence that directs secretion of the protein from the host cell. Both disclosed working examples require such leader sequences, as stated in the rejection, and it would appear that it is the use of such leader sequences that

distinguishes the claimed protein from that suggested by the prior art, as appellants are traversing the rejection under 35 U.S.C. §103 (a) on the basis that the prior art teachings of recombinantly producing hPTH in bacterial cells would not have resulted in the production of the currently claimed protein, but have failed to include any feature in the pending claims that is not taught by the prior art, namely the inclusion of the secretory leader sequence. In view of this, the Examiner maintains the position that the use of an appropriate secretory leader sequence appears to be essential to the claimed invention, and the claims are accordingly found to be incomplete for omitting such an essential element.

Appellants arguments of the rejection under 35 U.S.C. §102(b) or 103 over Brewer is found at page 7 of the appeal brief. Appellants argue therein that the Maggio declaration, originally submitted in 1996 (see paper number 13), states that the Kimura and Kumagaye publications, which were subsequent to the Brewer patent, “show that the purification protocols discussed in Brewer et al. result in impure materials.” This argument has been fully considered but is not deemed persuasive. At paragraph 9 of the Maggio declaration, the declarant states that Brewer et al. contains three amino acid sequencing errors, at positions 22, 28 and 30 of the protein, and that these errors show that Brewer et al. did not teach the production of an intact hPTH peptide. This argument has been fully considered but is not deemed persuasive because the Examiner disagrees with the factual analysis of the reference, and its relationship to the claims in question. First, and foremost, it is noted that the claims are not limited to any particular sequence, and that in fact, claim 35, read in light of the specification, is drawn to an hPTH having other than the native human sequence (see page 15 of the specification wherein it is disclosed that a mutation of the 26th amino acid from Lysine to Glutamine was necessary to achieve the desired expression of full-length hormone in yeast). Accordingly, the interpretation that a particular sequence must have been obtained to meet the limitations of the claims is not warranted. Second, it has not been established on the record that the difference in sequence

between Brewer et al. and appellants is due to sequencing error, as opposed to an actual difference in protein sequence. Third, even *if, in arguendo*, the differences are due to sequencing error, this is *not* proof that the protein was not 'intact'. There may be a variety of reasons that sequencing error may occur. The fact that Brewer sequenced beyond the positions of the supposed errors, to residue 34, would argue against an interpretation that the protein being sequenced was not 'intact'.

That is, if the errors were due to truncation at the positions of the errors, Brewer et al. would not have been able to continue obtaining sequence past those points. Fourth, since appellants argument implies that Brewer's protein could not have been of the same purity as appellants have obtained via recombinant production, and that conclusion is based upon Brewer's sequencing data, it is noted that the only portion of the specification which relates to purity of the protein is the disclosure that the protein was partially sequenced (page 7, starting at line 27), which the ordinary artisan would recognize as requiring a relatively pure preparation of the desired protein (although no exact percentage purity can be implied). Based upon the fact that the specification discloses obtaining the sequence of 19 and 45 amino acids respectively, from the yeast and E. coli-produced protein, Brewer's ability to obtain 34 amino acids would seem to indicate that comparable purity was achieved. Fifth, appellants argue that the lack of purity of the Brewer hormone "is evidence by Fig. 3 of Brewer et al.," citing PNAS 69:3585, published in 1972. It is noted that this reference was made of record by appellants in the information disclosure statement, paper number 25, and was also cited in the prosecution of the Brewer patent. This argument has been fully considered but is not deemed persuasive because the question at hand is not whether the protein of Brewer et al. was absolutely homogeneous, nor is it the skill or fastidiousness of Brewer et al. as sequencers (it is noted that minor peaks could be caused by a variety of factors, not limited to the contamination of the protein as alleged by appellants, such as contamination of the instruments being used for the Edman degradation, or alternatively might be due to electronic 'noise' or other instrument failure.). Rather, the question is whether or not the protein of Brewer et al. meets the limitations of the claims, which require that the protein be "substantially homogeneous", with "a purity of greater than 95%." As stated above, given that the only measure of purity that we have for the claimed protein seems to be

appellants ability to sequence 19 and 45 residues of the protein, and given that Brewer was able to sequence 34 residues, it appears that the purity of Brewer's protein was comparable to that obtained by appellants. It is noted that although appellants would argue that the presence of minor peaks on Brewer's mass spec. is evidence of lack of purity, that appellants have furnished no comparative evidence that such minor peaks are not obtained from the claimed protein.

At page 8 of the appeal brief, appellants argue that Brewer's intent was not to "identify, isolate and characterize an intact hPTH." This argument has been fully considered but is not deemed persuasive because Brewer's intent is irrelevant to the question of whether or not Brewer's protein anticipates the claimed invention. Appellants further argument (pages 8-9) that a single substitution can alter the biological properties of the hormone has been fully considered but is not deemed persuasive because (a) the claims are not limited to any particular sequence (as discussed above), (b) the claims have no limitation as to biological activity of the claimed protein, and (c) it has not been established of record that the hormone isolated by Brewer differs in any way from that being claimed.

Appellants argument at page 9 of the appeal brief (point C), has been fully considered of record but not deemed persuasive. With respect to this argument, the Examiner, in paper number 15 at page 7, stated:

The declaration by Dr. Maggio under 37 C.F.R. §1.132 is not persuasive to overcome this rejection. At paragraph 9, Dr. Maggio states that because Brewer et al. contains three errors in the amino acid sequence, that Brewer does not teach production of intact peptide. This argument has been fully considered but is not deemed persuasive because the protein purified by Brewer does indeed appear to have been intact, in the sense of not having been degraded or damaged. It cannot be concluded that a possible sequencing error indicates that the peptide was not "intact". The further argument of Brewer, pertaining to the purification of the synthesized 34 amino acid species is not relevant to the rejection, as Brewer was cited for the purification of naturally occurring hPTH, and not for the synthesis of the 34 amino acid fragment. Dr. Gautvik's declaration does not directly address this rejection.

Applicants argument that Brewer contains three incorrect amino acids in the disclosed sequence of the first 34 amino acids of the protein is not persuasive, both because the claims contain no limitation as to particular

5 sequence, and because, even if Brewer sequenced the protein incorrectly, the protein itself, which was obtained from the natural source, appears to meet the limitations of the claims. The additional arguments pertaining to Brewer are drawn to the *synthetic* peptide of Brewer, and do not address the purified (naturally occurring) protein disclosed by Brewer, upon which this rejection is based.

10 Appellants go on to argue that “A mistake in the N-terminal region of hPTH could change the activity of the C-terminal region as it could alter the reading frame or a binding epitope of hPTH.” This argument has been fully considered but is not deemed persuasive for two reasons. First, as repeatedly stated above, there is no limitation in the claims as to either sequence or activity of the claimed protein. Second, appellants argument confuses *nucleic acid* sequencing with *protein* sequencing. The sequence in question was determined directly by analysis of the *protein*, which was successively
15 degraded to release a single amino acid at a time from the amino terminus of the protein, and the identity of each such amino acid was determined. There is no issue of anything being *encoded* by the amino acid sequence, it is what it is. Appellants argument pertaining to alteration of reading frame would only be pertinent if the sequence of amino acids had been deduced by determining the sequence of the nucleic acid which *encodes* the protein. Nucleic acids which encode proteins have three
20 ‘reading frames’, as amino acids are each encoded by a three base-stretch of nucleic acid, called a ‘codon’ or a ‘triplet’. Thus, when sequencing nucleic acids, it is indeed possible that a single mistake in the beginning of the sequence can result in a ‘frame shift’, in which the protein encoded by the remainder of the molecule would have an entirely different sequence than if the mistake had not been made. However, there is no such issue when a protein sequence is directly determined from the
25 protein itself, and not from the nucleic acid which encodes it.

Appellants traversal of the rejection of claims 31-34 as being obvious under 35 U.S.C. §103 over Breyel or Sung or Mayer, any of the three in view of Kaisha et al. begins at page 9 of the appeal brief.

At page 10, appellants argue that Breyel et al. do not teach an intact and substantially homogeneous preparation of hPTH. This argument has been fully considered but is not deemed persuasive because it takes the reference in isolation, and not in the combination in which it was cited. As set forth in the rejection, Breyel's teaching of the recombinant production of hPTH in *E. coli*,
5 taken with Kaisha et al. teaching of the desirability of making large quantities of hPTH, and that the person of ordinary skill in the art, given a preparation containing hPTH, would be able to devise a protocol for purifying such with a reasonable expectation of success and without undue experimentation would have made it obvious to the person of ordinary skill in the art at the time the invention was made to express hPTH as taught by Breyel et al. and then to purify the hPTH so
10 produced as suggested by Kaisha et al. to obtain highly purified hPTH. The ordinary artisan would have been motivated to do so in view of the art recognized desirability of obtaining hPTH in pure form, as evidenced by all three cited references. The teachings of Kaisha et al. indicate that the ordinary artisan would have had at least a reasonable expectation of success at purifying hPTH once produced as taught and/or suggested by Sung or Breyel or Mayer. Although Breyel et al. recognized
15 that the protein had a short half-life when made in *E. coli*, such would not have precluded obtaining a homogeneous preparation of the protein, although perhaps not in great quantity. It would have been within the skill of the ordinary artisan to have separated the full-length (1-84) from the degraded forms of the product, to obtain the claimed homogeneous preparation. Appellants have not argued this point.

20 Appellants arguments of the remaining references are similarly directed at the references individually, rather than in the combination in which they were cited. In response to appellant's arguments against the references individually, one cannot show non-obviousness by attacking references individually where the rejections are based on combinations of references. *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co., Inc.*, 800 F.2d 1091, 231
25 USPQ 375 (Fed. Cir. 1986). Appellants arguments fail to address the rejection as made, which is on the basis that the three primary references render obvious the recombinant production of hPTH, and that taken in view of the secondary reference, Kaisha, one of ordinary skill in the art would have been

both motivated to and capable of isolating a pure preparation of recombinantly produced hPTH(1-84).

With respect to Sung et al., (page 10 of the appeal brief), appellants argue that Sung's suggestion that "a study is now conducted in the expression of these gene products" does not suggest appellants claimed invention, citing *In re Deuel* in support of the position. This argument has been fully considered but is not deemed persuasive because Sung's statement is a direct suggestion of expressing the protein which, taken with the other references such as Kaisha, provides motivation to make the protein in the manner suggested by Sung. This is *not*, as was the case in *Deuel*, an invitation to conduct future research, but rather a direct suggestion to perform the expression. The issue in *Deuel* was substantively different; *Deuel* was drawn to the obviousness of a DNA sequence encoding a protein which had been only partially characterized (an N-terminal sequence was known), and for which *no* corresponding nucleic acid (encoding the protein) had been previously isolated. In the instant case, Sung et al. teach the construction of vectors for the direct expression of hPTH in bacterial, specifically *E. coli*, cells. The requisite sequences were both known and disclosed. Appellants remaining argument of Sung et al. is not persuasive for reasons discussed above with respect to Breyel et al.

With respect to Mayer et al., appellants repeatedly allege that because Mayer et al. do not provide the specific results of their experiments, that is, the results of the radioimmunoassay that would have been used to confirm production of the protein, that they "do not provide any data to substantiate the claims of producing recombinant hPTH in *E. coli* and mammalian kidney cells." This argument has been fully considered but is not deemed persuasive because as stated in the rejection, Mayer et al. teach recombinant production of hPTH in *E. coli*, see page 9, first full paragraph for example. The protein was purified from the cells and shown to be biologically active. Appellants protest that insufficient data were shown is insufficient to support an assertion that the recombinant production reported by Mayer et al. did not occur. Appellants have failed to support the assertion by facts or reasoning. As was found above with respect to the Breyel et al. reference, even *if* the recombinant product were not particularly stable, this would not render it either impossible, or even

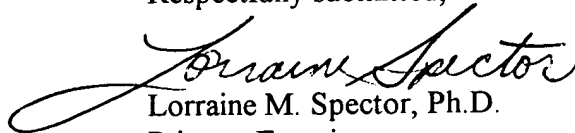
require undue experimentation to obtain a product consistent with the rejected claims, in view of the state of the art, as evidenced by Kaisha et al. Merely because a protein may be degraded does not mean that it would require undue experimentation to purify a 'substantially homogeneous' preparation of full-length protein. The problem of protein degradation is one that is commonplace in the art of protein purification (as *all* cells comprise proteases, and those proteases must be dealt with in any protein purification, although particular proteins may be more or less susceptible to such), and it is well within the skill of the person of ordinary skill in the art to obtain a homogeneous preparation of a full-length protein.

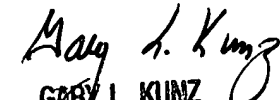
Finally, appellants argue that Kaisha do not teach an "essentially homogeneous" hPTH protein. This argument has been fully considered but is not deemed persuasive because once again, appellants are arguing the reference individually, rather than in the combination in which it was cited. Kaisha et al. were not cited for teaching an "essentially homogeneous hPTH" (it is noted that the claims are to a *substantially* homogeneous preparation), but rather to establish that it would have been well within the skill of the person of ordinary skill in the art to purify the protein produced as suggested by any of the primary references. Kaisha et al. suggest a number of specific, readily available procedures that would reasonably be expected to produce a homogeneous preparation of protein, including conventional procedures such as salting-out, dialysis, filtration, centrifugation, concentration and lyophilisation and further teach that a preparation of the highest purity can be obtained by the above-mentioned techniques in combination with other conventional procedures such as adsorption and desorption with ion exchange, gel filtration, affinity chromatography, isoelectric point fractionation and electrophoresis. Appellants have not presented any reasoning, fact or evidence to support the assertion that following Kaisha's teachings of the desirability of obtaining hPTH and suggestions as to how to do so would not, in combination with the various recombinant production methods of the three primary references, result in obtaining a protein consistent with the claims.

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Art Unit: 1646


For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,


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Molecular Biology and Radiochemical Assays of Calcitropic Hormones

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THE HUMAN CALCITONIN GENE - R.K. Craig

Studies on the structure and expression of the human calcitonin gene, demonstrate that in common with the rat calcitonin gene, that differential usage of exons in an apparently tissue specific manner results in the expression of mRNA species encoding the calcitonin or the calcitonin gene related peptide (hCGRP). Our studies using sequence specific cDNA hybridisation probes, nucleotide sequence analysis and poly(A)-containing RNA isolated from medullary thyroid carcinoma tissue and small-cell carcinoma cell-lines, point to the involvement of two separate post-transcription mechanisms in the differential expression of this gene. A post-transcriptional cleavage event leading ultimately to the production of calcitonin mRNA, or a selective use of splice acceptor sites leading to the synthesis of CGRP mRNA. The availability of cell-lines synthesising predominantly calcitonin or CGRP mRNA should permit further studies which will provide insight into the regulation of these post-transcriptional events.

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Complementary DNA copies of human preproparathyroid hormone (preproPTH) mRNA were cloned in *E. coli* after insertion of double-stranded DNA into the Pst I site of plasmid pBR322 using the poly (dG), poly (dC) homopolymer extension technique (Hendy et al, PNAS, 78, 7365, 1981). Recombinant plasmids coding for preproPTH were identified by filter hybridisation assay using as a probe ³²P-labelled bovine preproPTH cDNA. Nucleotide sequence analysis of five recombinant plasmids permitted the assignment of 74 nucleotides of the 5' noncoding region, the entire coding region of 345 nucleotides and the entire 3' noncoding region of 348 nucleotides of the mRNA. The coding sequence predicted the previously unknown 'pre' amino acid sequence and clarified the hormone's amino acid sequence. The 5' noncoding region contains an AUG codon followed by a UGA stop codon before the authentic initiator codon. The 3' noncoding region contains two AAUAAA sequences, potential signals for polyadenylation.

Recombinant phage containing the gene encoding human preproPTH were isolated from a lambda phage gene library (Vasicek et al, PNAS, 80, 2127, 1983). The human preproPTH gene contains two intervening sequences that separate the gene into a 5' noncoding domain, a 'prepro' sequence domain, and a domain containing the PTH sequence and the 3' noncoding region. The gene is approximately 4,200 base pairs long. Restriction endonuclease analysis of human leukocyte DNA showed that the haploid human genome contains one copy of the preproPTH gene. The gene has been mapped to the short arm of chromosome 11 using human-rodent cell hybrids and Southern filter hybridisation of cell hybrid DNA (Naylor et al, Som. Cell. Gen. 9, 609, 1983).

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In conclusion Ca exerts its action by a dual mechanism a rapid action at the posttranscriptional level and a delayed one involving the transcriptional level.

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To study the secretion of human parathyroid hormone (hPTH) in *E. coli* and yeast, we have constructed vectors which are designed to express efficiently in each of these organisms the complementary DNA (cDNA) for human preproPTH (hppPTH).

DNA sequences encoding a lac promoter, the prepro sequence of hppPTH, and the enzymatically active carboxy terminal fragment of *E. coli* β-galactosidase were fused together on an *E. coli* plasmid. In high producers of β-galactosidase activity, the coding sequence of that enzyme was replaced by the coding sequence of hPTH. In various *E. coli* strains we identified three proteins which were immunoprecipitable with human PTH antiserum and had the appropriate size and aminoterminal sequence of hppPTH, and of peptides beginning at amino acids 3 and 8 of intact hPTH. None of them appeared soluble in the periplasmic fraction. In the yeast system, we inserted hppPTH-cDNA between the promoter and 5' non-coding region of yeast pyruvate kinase and a sequence directing termination of transcription from the yeast triose phosphate isomerase gene. This fusion gene was then cloned into a plasmid containing a yeast origin of replication and a selectable marker. In two independent clones which survived selection we have immunoprecipitated a protein with the size and aminoterminal sequence of hppPTH. It was localized inside the cell.

In conclusion, our data show that PTH-related peptides encoded by human preproPTH cDNA can be expressed in *E. coli* and yeast but are not secreted.

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STRUCTURAL ANALYSIS OF THE HUMAN PARATHYROID HORMONE GENE. Henry M. Kronenberg, Thomas J. Vasicek*, Geoffrey M. Hendy*, Alexander Rich*, and John T. Potts, Jr., Endocrine Unit, Massachusetts General Hospital, Boston, MA., and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA.

We are comparing the structures of the gene and the messenger RNA (mRNA) coding for human preproparathyroid hormone (preproPTH). This comparison will allow us to define sequences controlling the initiation and termination of RNA transcription, and will also allow us to locate so-called intervening DNA sequences; that is, sequences of DNA that interrupt the PTH mRNA and are removed from an mRNA precursor before mRNA is released from the cell nucleus. The human preproPTH gene was isolated from a lambda phage human gene library. We have subcloned portions of the 14,000 base pair human DNA into the plasmid pBR322 in order to facilitate sequence analysis. We used the agarose gel transfer method of Southern to identify fragments of the human DNA containing PTH information. Human DNA fragments were separated by agarose gel electrophoresis and then transferred to nitrocellulose paper. PTH-related fragments were recognized by hybridizing the nitrocellulose-bound DNA to radioactive DNA derived from a clone containing human PTH mRNA sequences. The relevant fragments were then re-cloned into pBR322. Our analysis so far shows that no intervening sequence interrupts the gene in a 215 pair region extending from amino acid 28 of PTH through the rest of the coding region and including the first 38 nucleotides of the 3'-noncoding region of the mRNA. Since intervening sequences often separate functionally important domains, we might have expected an intervening sequence separating the biologically active 1-34 region from the distal half of PTH. Further restriction endonuclease and DNA sequence analysis will allow us to complete the characterization of the gene.

HUMAN PARATHYROID CARCINOMA CELLS THAT PRODUCE PTH: LONG TERM MAINTENANCE IN TISSUE CULTURE. J. Lemann Jr., R.A. Patrillo*, A.C.F. Ruckert*, S.W. Wilson*, R.O. Hruska* and R.W. Gray, Departments of Medicine, Gynecology/Obstetrics, Surgery and Biochemistry, Medical College of Wisconsin, Milwaukee, WI.

Segments of metastatic parathyroid carcinoma from a patient with a serum Ca of 17.2 mg/dl, P 1.6 mg/dl and iPTH 160 uEq/ml (normal 2-10 uEq/ml; Slatopolsky COOH and intact antibody; 1 uEq/ml = about 40 pg bPTH/ml) were explanted into tissue culture 4/23/78 using 50% Waymouth's 752/1, 30% Gey's balanced salt solution and 20% human cord serum as medium. Initial epithelial cell density was estimated at about 6×10^6 cells/flask. Cell proliferation was seen within 4 days and persists. However by 11/79, 1 1/2 years after the cultures were begun epithelial density decreased to about 10^3 to 10^4 cells/flask where it has remained. The initial culture fluid iPTH of 1.5×10^5 uEq/ml or 6 μ g bPTH equivalent/ml has declined in parallel to the decrease in cells and has remained at about 100 uEq/ml or 40 ng bPTH/ml, for the past year. Estimated PTH production has remained at about 1 pg/cell/day or 100 attomol/cell/day for 2 1/2 years. Gel filtration of early (5/78) and late (3/80) culture fluids have shown that the peak of iPTH comigrates with 125I-bPTH without smaller fragments of iPTH. Biological activity of early culture fluid PTH was tested in the fetal rat bone system by Dr. Paula Stern, Northwestern University. Bone resorbing activity was simply additive to that of synthetic bPTH 1-34 and was present at a concentration of 6 μ g bPTH equivalent/ml, similar to that observed as iPTH. Attempts to stimulate cell proliferation with several growth factors or lowering the medium Ca from 1.2 to 0.8 mM has thus far failed. We conclude that these human malignant parathyroid cells continue to produce intact and biologically active hPTH after 2 1/2 years in tissue culture.

ON THE RELATIONSHIP OF HYPERPARATHYROIDISM AND SERUM GLYCOPROTEINS AND SIALYL TRANSFERASE. A.A. Elcata and L. Sheeler*, University of Rochester, Rochester, N.Y. and Cleveland Clinic, Cleveland, Ohio

Although parathyroid hormone (PTH) is metabolized by the liver, it is still unclear whether the hormone affects hepatic function. To study this, we investigated whether serum glycoproteins and sialyl transferase activity (STA), markers of hepatic function, were altered in hyperparathyroidism (HPT). Serum from normal (n=16) and (HPT) (n=20) subjects were analyzed for total precipitable (ppt) and soluble (serumucoid) sialic acid, hexosamine, neutral hexoses, fucose, uronic acid, and protein and for specific glycoproteins by radial immunodiffusion. (STA) was measured by the incorporation of isotopic sialic acid into desialated fetuin. Initial studies showed that more proteins (40%) from (HPT) sera were retained on Con-A-agarose than from normal or Pagetic sera and were eluted with methyl-D-mannoside. Total (ppt) carbohydrate content was increased. The increase was greatest for sialic acid and hexosamine ($p < 0.01$). (ppt) hexoses correlated with increased PTH ($r=0.64$) and alkaline phosphatase ($r=0.52$). Serumucoid and uronic acid were unchanged. Specific glycoproteins were either decreased ($p < 0.05$) (transferrin, a-2-macroglobulin) or unchanged (haptoglobin, a-1-antitrypsin). (STA) was similar in (HPT) and normals but correlated ($p < 0.05$) with PTH ($r=0.68$), (ppt) hexoses ($r=0.60$), and alkaline phosphatase ($r=0.60$) in the (HPT). We concluded (1) that (HPT) was associated with increased total carbohydrate content of serum proteins but not necessarily specific glycoproteins, (2) that its disease activity, as noted by changes in PTH and alkaline phosphatase, was reflected by changes in (STA) and (ppt) hexoses, and (3) that there was an unexplained relationship between hepatic glycoprotein metabolism and (HPT).

PROLACTIN-STIMULATED PARATHYROID HORMONE SECRETION IS NOT MEDIATED BY CYCLIC AMP OR CATECHOLAMINES. L. Magliola*, M.L. Thomas and L.R. Forte. Dept. of Pharmacology, Univ. Missouri and Truman VA Hospital, Columbia, Mo.

Recent reports suggest that bovine parathyroid glands contain dopamine (DA), possibly within mast cells, and that DA synthesis and release from rat brain is stimulated by prolactin (PRL). We previously reported that PRL stimulates PTH secretion in dispersed bovine parathyroid cells. The present study was undertaken to explore the possibility that PRL-stimulated PTH release is mediated via endogenous catecholamines. PTH secretion due to PRL was not blocked by either the β -adrenergic or dopaminergic antagonists, propranolol and fluphenazine, respectively. PRL did not increase cyclic AMP above basal levels in parathyroid cells incubated for up to 3 hours with or without 1 mM MIX. DA- and isoproterenol (ISO)-stimulated cyclic AMP were effectively blocked by fluphenazine and propranolol, respectively. These data suggest that PRL-stimulated PTH release is not mediated through the β -adrenergic or dopaminergic receptor systems of parathyroid cells and confirms our previous report that the effect of PRL is not mediated by cyclic AMP. We also found that haloperidol, a DA-antagonist, markedly stimulated PTH release. Haloperidol in combination with maximal levels of DA, or PRL produced additive increases in PTH secretion. Also, haloperidol-stimulated PTH secretion was not blocked by the antagonists, propranolol, phentolamine or atropine. Other DA-antagonists such as fluphenazine and butaclamol did not stimulate secretion. In conclusion, the results of this study suggest that PRL and haloperidol may stimulate PTH secretion through agonist-like activity via receptors other than the β -adrenergic or dopaminergic receptor types.

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Nucleotide sequence of the human parathyroid hormone gene

(Z-DNA/ π VX plasmid/intervening sequence/domain evolution)

THOMAS J. VASICEK^{*†‡}, BARBARA E. MCDEVITT^{*†§}, MASON W. FREEMAN^{*†}, BARBARA J. FENNICK^{*†}, GEOFFREY N. HENDY^{*†¶}, JOHN T. POTTS, JR.[†], ALEXANDER RICH^{||}, AND HENRY M. KRONENBERG^{*†}

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Contributed by Alexander Rich, December 29, 1982

ABSTRACT From a λ phage gene library we have isolated phage containing the gene encoding human preproparathyroid hormone. The phage were isolated by using both the plaque-hybridization technique and the *in vivo* recombination-selection technique. The human preproparathyroid hormone gene contains two intervening sequences that separate the gene into a 5' non-coding domain, a "prepro" sequence domain, and a domain containing the parathyroid hormone sequence and the 3' noncoding region. The gene is approximately 4,200 base pairs long. Restriction endonuclease analysis of human leukocyte DNA shows that the haploid human genome contains one copy of the preproparathyroid hormone gene. A 14-base-pair sequence of alternating purines and pyrimidines that has the potential of adopting the Z-DNA conformation lies 134 base pairs upstream from the presumed site of initiation of transcription.

Parathyroid hormone (PTH) is the principal homeostatic regulator of blood calcium, and, in turn, the secretion of PTH is closely regulated by the blood level of calcium. While the regulation of secretion of PTH has been studied extensively, the regulation of the synthesis of PTH has received comparatively little attention. We (1) and others (2, 3) have cloned cDNA encoding bovine PTH. More recently, we have cloned cDNA encoding human PTH as well (4). Here we describe the isolation and DNA sequence analysis of genomic DNA encoding human PTH. The human PTH gene contains two intervening sequences that separate the mRNA sequence into three functional domains. DNA blotting experiments show that the haploid human genome contains only one PTH gene.

METHODS

Screening a λ Phage Library. A human gene library in phage Charon 4A, derived from fetal liver, was provided by T. Maniatis (5). The library was first screened by the procedure of Benton and Davis (6), using plasmid pPTHm113 (4) containing human PTH cDNA as a nick-translated (7) probe. The library was subsequently screened by the method of Seed (8). Plasmid DNA was isolated from *Escherichia coli* strain W3110r⁻m⁺(p3)(π VX) by an alkaline miniprep procedure (9), the DNA was electrophoresed through a 0.7% agarose gel, and the π VX plasmid was isolated from the gel. By using phage T4 DNA ligase, the *Bgl* II/*Xba* I fragment from pPTHm122 (4) was inserted into the corresponding sites on the π VX plasmid. The resultant plasmid, π VX-PTH, containing the PTH cDNA linked to the selectable marker *supF*, was introduced into *E. coli* strain W3110r⁻m⁺(p3). One million library phage were then amplified on one plate of the resultant strain W3110r⁻m⁺(p3)(π VX-PTH). Then 5×10^8 of the resultant phage were grown on one plate of strain W3110r⁻m⁺ Su⁻, a strain containing no amber suppressor tRNA gene. Growth of Charon 4A phage on *E. coli* W3110r⁻m⁺ Su⁻

selected for phage that had incorporated the *supF* gene, encoding an amber suppressor tRNA, from the π VX-PTH plasmid. All work with organisms containing recombinant DNA was performed in a P1 physical containment facility according to the then current guidelines of the National Institutes of Health.

Subcloning PTH Gene Fragments. Fragments of DNA generated by restriction enzyme digestion of λ hPTH1 and λ hPTH2 were subcloned in plasmid pBR322, after blot-hybridization analysis (10) was used to determine which fragments contained portions of the PTH gene. Fragments resulting from cleavage with one restriction endonuclease were ligated to DNA from plasmid pBR322 that had been cleaved with the same enzyme and treated with calf intestinal alkaline phosphatase to prevent intramolecular religation. Fragments resulting from cleavage with two restriction endonucleases were ligated with the appropriate fragment of DNA from pBR322, which had also been cleaved with those enzymes. DNA fragments were isolated from agarose gels by using glass powder to bind the DNA (11) and were ligated by using T4 DNA ligase.

DNA Sequence Analysis. All DNA sequences were determined by the chemical method of Maxam and Gilbert (12). DNA was end-labeled either with polynucleotide kinase or with the large fragment of DNA polymerase I.

Blot-Hybridization of Unc cloned Human DNA. DNA was isolated from blood leukocytes from 50 ml of blood from a normal volunteer (13). DNA was digested to completion with 10-fold excess enzyme, according to suppliers' protocols. Completion of the digestions was assayed by the addition of small amounts of an appropriate plasmid to an aliquot of the reaction and electrophoretic analysis of the products of digestion of the aliquot. Then 10 μ g of digested DNA was electrophoresed through a 1.0% agarose gel and transferred to nitrocellulose. Hybridization was performed according to a dextran sulfate protocol (14), using purified PTH cDNA fragments as probes. Nick-translation was performed with all four α -³²P-labeled deoxyribonucleoside triphosphates; the resultant DNA contained 10⁸ cpm/ μ g. The intensities of autoradiographic bands were measured with a Joyce-Loebl microdensitometer. Calculations of the number of copies of the PTH gene in the human genome used 3.3×10^9 base pairs (bp) as the size of the haploid human genome (15), 44,000 bp as the size of λ hPTH1, and 43,000 bp as the size of λ hPTH2.

RESULTS

Isolation of λ Phage Containing the Human PTH Gene. We first screened a human fetal liver genomic DNA library constructed in phage Charon 4A by Lawn *et al.* (5). We used the plaque-hybridization method, with nick-translated plas-

Abbreviations: PTH, parathyroid hormone; bp, base pair(s).

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mids pPTHm113 and pPTHm122 (4) as probes. Only one plaque, λ hPTH1, was positive. A partial physical map of λ hPTH1 was constructed by performing blot-hybridization analysis of λ hPTH1 DNA cleaved with a variety of restriction endonucleases and probed with specific DNA fragments from pPTHm122. This analysis demonstrated that λ hPTH1 contained the PTH gene's coding region and 3' noncoding region but that the 5' noncoding region was missing from the phage.

We chose to rescreen the λ phage library by using the recombination-selection method of Seed (8). We isolated from pPTHm122 the 288-bp *Bgl* II/*Xba* I fragment, which contains the PTH coding sequence and part of the mRNA's 3' noncoding region. This fragment was inserted into the plasmid π VX, using the *Bgl* II and *Xba* I sites in the "polylinker" region of that plasmid. Following Seed's protocol, we passed 10^6 phage from the gene library through *E. coli* strain W3110r⁻m⁺(p3) that had been transfected with π VX-PTH. Then 5×10^6 of the resultant phage were plated on *E. coli* W3110r⁻m⁺ Su⁻ cells, which do not contain an amber suppressor tRNA gene and therefore do not allow the production of Charon 4A phage. Sixty plaques survived that selection. Six of the 60 plaques were screened by restriction enzyme digestion analysis and found to be identical. One plaque, λ hPTH2, from among these 6 was chosen for further analysis. Digestion of λ hPTH2 DNA with restriction endonucleases and subsequent blot-hybridization experiments using fragments of pPTHm122 as probes demonstrated that λ hPTH2 contained the entire human PTH gene with the π VX-PTH plasmid inserted, as expected, into the region between the *Bgl* II and *Xba* I sites used in constructing π VX-PTH (Fig. 1).

Examination of the Human PTH Gene in Unc cloned DNA. In order to verify that the two λ phage that we had isolated contained the normal human PTH gene free of artifactual rearrangements introduced during cloning, we compared the sizes of DNA fragments generated by restriction endonuclease digestion of human leukocyte genomic DNA with the sizes of corresponding fragments from λ hPTH1 and λ hPTH2. After agar-

ose gel electrophoresis and transfer to nitrocellulose, DNA fragments were hybridized with probes specific for either the 5' end or the 3' end of the human PTH gene. We used the 2,150-bp *Hind*III fragment which includes the first exon and part of IVS1 from pPTHg105 (see Fig. 1) as the 5'-specific probe. An 800-bp *Hpa* II fragment of pPTHm122 (4) was used as a 3'-specific probe. This fragment contains 100 bp of pBR322 as well as 547 bp of DNA corresponding to the second exon and part of the third exon. It also contains 49 bp at the end of the first exon; this short sequence does not form detectable hybrids under the conditions of hybridization used here. Fig. 2 demonstrates the comigration of the resultant radioactive fragments from the λ phage and leukocyte DNA. Further, because no unexpected bands appeared in the lanes containing leukocyte DNA, the results suggest that the human PTH gene is represented just once in the haploid human genome. To confirm this suggestion, we measured the intensities of the PTH-specific bands on the autoradiograms and compared them with the intensities of the bands generated from known amounts of DNA from λ hPTH1 and λ hPTH2. With the 5'-specific PTH probe, this analysis yielded 1.0 PTH gene per haploid genome; with the 3'-specific PTH probe, the result was 0.6 PTH gene per haploid genome.

DNA Sequence Analysis of the Human PTH Gene. Restriction endonuclease analysis and gene blotting experiments suggested that two intervening DNA sequences interrupted the PTH gene, one approximately 3,400 bp in length, and the other one about 100 bp in length. We determined the entire sequence of the gene, excluding the sequence of the internal portion of the first intervening sequence, and, in addition, determined the sequence of several hundred base pairs of DNA flanking the PTH gene. Fig. 1 illustrates the restriction enzyme map of fragments of λ hPTH1 and λ hPTH2 subcloned in pBR322 to facilitate sequence analysis, and also indicates the sequencing strategy. Fig. 3 illustrates the DNA sequence.

DISCUSSION

DNA Sequence Data. Fig. 1 shows that most of the sequence depicted in Fig. 3 was determined by analyzing both strands of

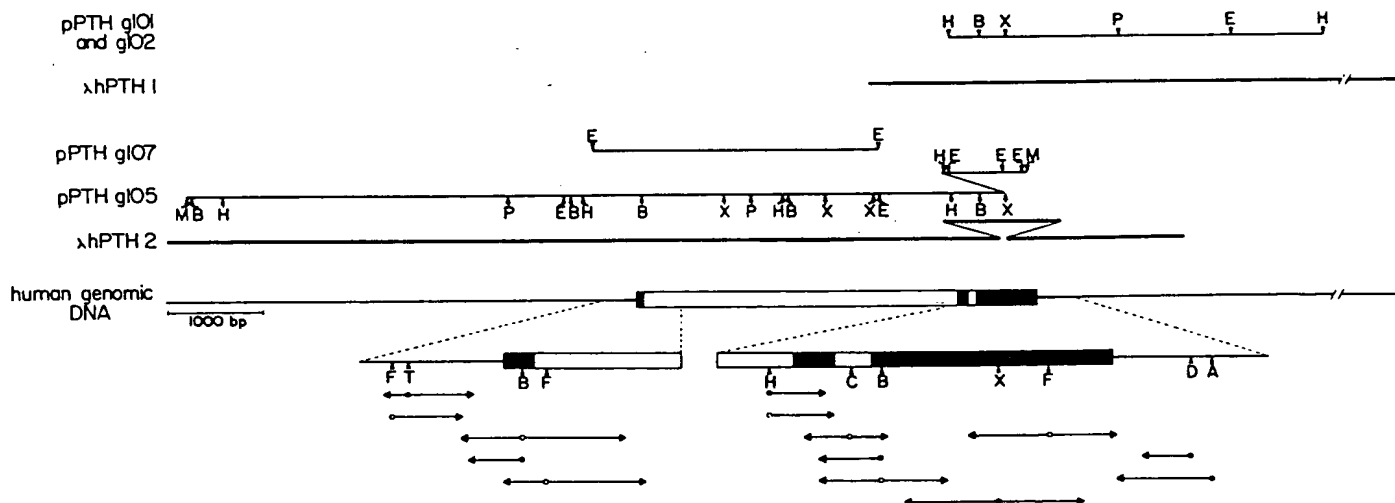


FIG. 1. Physical map of the human PTH gene. Horizontal lines indicate the length of human DNA inserted into the indicated λ phage and plasmid subclones. The raised portion of λ hPTH2 indicates the π VX-PTH insert. pPTHg101 and pPTHg102 were constructed by inserting the indicated fragment of λ hPTH1 into *Hind*III-cleaved pBR322. They differ only by their orientation in pBR322. pPTHg105 and pPTHg107 were constructed by inserting the indicated fragments of λ hPTH2 into *Bam*HI-cleaved and *Eco*RI-cleaved pBR322. Restriction enzyme maps of the human portions of pPTHg101 and pPTHg105 are indicated. The human PTH gene is indicated on the line "human genomic DNA" by the thick line; black areas are exons, white areas are introns. More detailed and magnified copies of portions of the PTH gene are given below the human genomic DNA line. Arrows at the bottom of the figure indicate the DNA sequence analysis strategy. Open circles represent 5' ends of fragments labeled with polynucleotide kinase. Closed circles represent 3' ends of fragments labeled with the large fragment of DNA polymerase. Arrows show how far the DNA sequence could be reliably read without reference to other data. The broken line at the end of λ hPTH1 indicates that 9,000 bp have been deleted from the figure for ease of presentation. A, *Ava* II; B, *Bgl* II; D, *Dde* I; E, *Eco*RI; F, *Hinf*I; H, *Hind*III; M, *Bam*HI; P, *Hpa* I; X, *Xba* I.

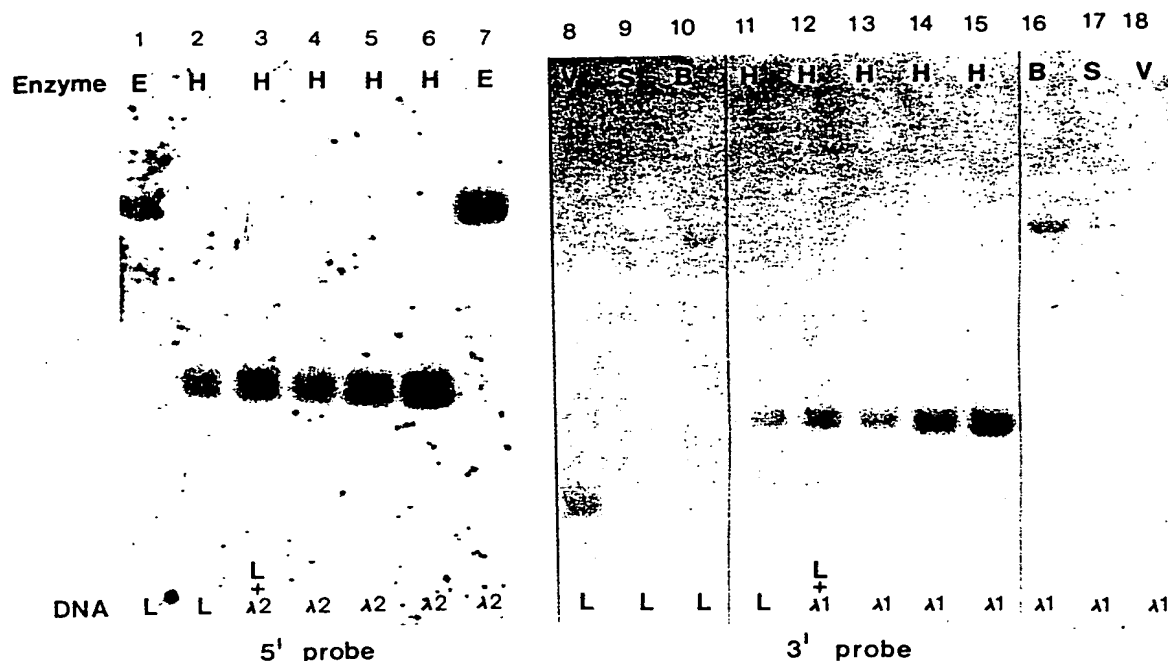


FIG. 2. Blot-hybridization analysis of human leukocyte DNA and cloned DNA. High molecular weight leukocyte DNA (L) and DNA from λ hPTH1 (A1) and λ hPTH2 (A2) were digested with a series of restriction endonucleases (E, *EcoRI*; H, *HindIII*; V, *Pvu II*; S, *Sst I*; B, *Bgl II*) and applied to wells as indicated in the figure. Wells 1-7 were probed with the 5'-specific probe; wells 8-18 were probed with the 3'-specific probe. Ten micrograms of leukocyte DNA or 300 pg of phage DNA was applied to each well except for the following wells: wells 3 and 12, 10 μ g of leukocyte DNA plus 150 pg of phage DNA; wells 4 and 13, 150 pg of phage DNA; wells 6 and 15, 450 pg of phage DNA.

plasmid DNA and that the ordering of fragments generated by cleavage with restriction endonucleases was confirmed by sequencing across cleavage sites. The sequence of the human PTH mRNA predicted by analysis of the cloned genomic DNA confirms without exception the sequence determined from analysis of five cDNA clones (4). We are thus quite confident of the accuracy of the AUG sequence found in the 5' noncoding region of the mRNA sequence, for example. Further, the genomic DNA sequence confirms the sequence of the region encoding human preproPTH. This sequence, derived from a presumably normal fetal liver, allows us to deduce the human PTH sequence from an individual with no parathyroid gland disease. [Both the previously determined protein sequences (16) and cDNA sequence (4) were derived from parathyroid tumor materials.] As expected, we can conclude that patients with hyperparathyroidism make a structurally normal parathyroid hormone.

We have not determined the start point of PTH gene transcription either by using an *in vitro* transcription system or by analyzing the far 5' terminus of the human PTH mRNA. Consequently, the assignment of the transcription start site at nucleotide -3,566 in Fig. 3 is only an approximation based on three considerations: (i) The bovine PTH mRNA, whose 5' noncoding region closely resembles the 5' noncoding region of the human mRNA, begins at three tightly clustered A residues (corresponding to nucleotides -3,568, -3,566, and -3,561 of the human gene) as determined from reverse transcription of mRNA by Weaver et al. (3). (ii) Nucleotide -3,566 is 29 nucleotides from the sequence T-A-T-A-T-A, commonly found 27-31 nucleotides from transcription start sites (17). (iii) Transcription usually starts with an A (17), thus making nucleotide -3,568, a G residue in the human sequence, a less likely start site.

Organization of Intervening Sequences in the Human PTH Gene. By comparing the sequence of human PTH cDNA (4) and the sequence of human PTH genomic DNA, we can deduce the location of two intervening DNA sequences interrupting the

PTH gene. The first is approximately 3,400 bp long and interrupts the 5' noncoding region of the mRNA five nucleotides before the start of the coding region. The second intervening sequence is 103 bp long and comes between the second and third nucleotide encoding lysine-29 of the prepro-PTH molecule. Because of redundant sequences at the splice junctions, the exact splice start and stop sites cannot be assigned unambiguously. However, if we require that the splice donor and acceptor sequences follow the consensus sequence pattern of other donor and acceptor sequences (17), then the junctions can be assigned without ambiguity. In both introns the sequences of the donor and acceptor junction closely follow the consensus sequence predicted from previous analyses.

The first intervening sequence comes close to the end of the 5' noncoding region of the mRNA and therefore follows the pattern noted by Gilbert (18), that intervening sequences often separate mRNAs into functional domains. The exact location of the intervening sequence is intriguing, because it comes precisely after the potential AUG codon in the 5' noncoding region of the mRNA. After removal of the intervening sequence, the AUG is then followed by a UGA termination codon in the mRNA. The location of the AUG just before the start of the intervening sequence raises the possibility that alternative splicing patterns might allow the AUG to direct the synthesis of a second protein encoded by another nucleotide sequence. The first intervening sequence contains an in-frame termination codon only 15 codons after the AUG; therefore, an unspliced RNA could not use the AUG effectively. The possibility remains that the splice donor sequence could ligate to more than one acceptor sequence, however. Of course, the juxtaposition of the unusual 5' noncoding AUG and the intervening sequence may be coincidental. The bovine PTH mRNA has the sequence GUG instead of AUG in the 5' noncoding sequence (1, 3), suggesting that the 5' noncoding AUG is not of vital significance. On the other hand, it is striking that two of the other rare cases of mRNAs with 5'


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-3840
: : : : : : : : : : : : : : : :
gattcattaatcccatagaaatcttctgatggataattctgtatttgtaaaagtctttgcataagcccttgcagcccaatgctgttttcttttagtatccaattatctgaan

-3720
: : : : : : : : : : : : : : : :
cttaagaagagtgtgcaccgcccaatgggtgtgtgtatgtgtgctttgaacctatagttgagatccagagaattggagtgacatcatctgtaacataaagagcctctcttggttag

-3600
: : : : : : : : : : : : : : : :
cagaagacctatataaaagtaccatttaagggtctgcAGTCCAATTTCACAGTTGCTTTAGTTTACTCAGCATCAGCTACTAACATACCTGAACGAAGATCTTGTCTAAGACAT

-3480
: : : : : : : : : : : : : : : :
TGTATGgtaangtaaaacttaaaatcacttctgaatctcatgagattttgataatcaagttattatttaagtgtgtaccatttctacaantaccatgtttgtttcttcaaggtaaaatgcta

-3360
: : : : : : : : : : : : : : : :
agaagtttgagttatgtttaatatanaatgccacatacaaaaataa--- IVS 1
IS APPROXIMATELY
3400 BASE PAIRS LONG ---aagcttctcgtgaaaaccaaccaattagtttagtatgtcattct

-60
: : : : : : : : : : : : : : : :
gtgtactatagtttggaaatattaaaaatattttaaaataccctccattttgcttatccttttagTGAAG ATG ATA CCT GCA AAA GAC ATG GCT AAA GTT ATG ATT GTC
Met Ile Pro Ala Lys Asp Met Ala Lys Val Met Ile Val

40
: : : : : : : : : : : : : : : :
ATG TTG GCA ATT TGT TTT CTT ACA AAA TCG GAT GGG AAA TCT GTT AA gtaagtactgttttgccttggaaattggatttttaagtgtgactttatcatttcgaag
Met Leu Ala Ile Cys Phe Leu Thr Lys Ser Asp Gly Lys Ser Val Lys

150
: : : : : : : : : : : : : : : :
tggggagctaatgggaagtggccctctctgtttctcttcttcccgA AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT CTG AAC
Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn

250
: : : : : : : : : : : : : : : :
TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT CCC AGA GAT GCT
Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro Leu Ala Pro Arg Asp Ala

340
: : : : : : : : : : : : : : : :
GGT TCC CAG AGG CCC CGA AAA AAG GAA GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA GAC AAA GCT GAT GTG AAT
Gly Ser Gln Arg Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala Asp Lys Ala Asp Val Asn

430
: : : : : : : : : : : : : : : :
GTA TTA ACT AAA GCT AAA TCC CAG TGA AAATGAAAACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTCAAAGCTCTATTAAGAT
Val Leu Thr Lys Ala Lys Ser Gln ***

540
: : : : : : : : : : : : : : : :
TTCCAAGTGCCAATATTTCTGATATAACAACTACATGTAATCCATCACTAGCCATGATAACTGCAATTTTAATTGATTATTCTGATTCCACTTTTATTCATTGAGTTATTTTAATTAT

660
: : : : : : : : : : : : : : : :
CTTTTCTATTGTTTATTCTTTTAAAGTATGTTATTGCATAATTATATAAAGAATAAAATTGCACTTTAAACCTCTCTTCTACCTTAAATGTAAACAAAAATGTAATGATCATAAGT

780
: : : : : : : : : : : : : : : :
CTAAATAAATGAAGTATTTCTCACTCAttgcaagtatatcttttgggtatcactgatccacatgtttacattgatcatgactaggtagaacatacaagatttttttagtcatgt

900
: : : : : : : : : : : : : : : :
gtttcacatttggatatgttgacatcaacgttttagtattaccaagtatttaggtttccaantcttcaactagctcaantactgttgccttttgggttcaggaaaggaataaaatgctc

1020
: : : : : : : : : : : : : : : :
agcnaaaaaagggggcataaaagtggacc

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FIG. 3. DNA sequence of the human PTH gene. Nucleotides found in mature messenger RNA are capitalized; nucleotides in flanking and intervening DNA sequences are in lower case. Because of uncertainty about the start site of transcription and the exact length of the first intervening sequence, the first nucleotide of the coding region is designated nucleotide 1. The amino acid sequence of human preproPTH is indicated.

noncoding AUGs—rat liver and salivary gland amylase mRNAs—both have intervening sequences starting immediately after the 5' noncoding AUGs (19). In each case, in the mature mRNA, the AUG is followed by the potential codon AAA and then by the termination codon UAA. Further experiments will be required to evaluate the functional implications of these coincidental findings.

The PTH gene's second intervening sequence interrupts

preproPTH's "pro" sequence. PreproPTH contains 115 amino acids—a typical 25-amino-acid signal or "pre" sequence is followed by the 6-amino-acid "pro" sequence, Lys-Ser-Val-Lys-Lys-Arg, and then by the 84-amino acid hormone, PTH. The function of the "pro" sequence is unknown (see ref. 20 for discussion). Presumably the two basic residues, Lys-Arg, which are analogous to the two basic residues at the ends of most "pro" sequences, direct a peptidase to cleave the "pro" sequence from

PTH. Comparison of the "pro" sequences of the pro-PTH molecules from several species reveals that none of the first four amino acids of the "pro" sequences is conserved; in contrast, the last two residues are always Lys-Arg (21). The human PTH gene's second intervening sequence, then, separates the "pre" sequence and the variable portion of the "pro" sequence from the basic residues Lys-Arg and the PTH sequence. According to Gilbert's hypothesis, through evolutionary time, the PTH sequence carries with it the two basic residues required for cleavage of the hormone sequence from any peptide to which splicing events might fuse it.

Number of PTH Genes. Comparison of the sizes of DNA fragments generated by restriction enzyme digestion of uncloned human DNA and DNA from λ hPTH1 and λ hPTH2 suggests that major rearrangements did not occur during the cloning. Two kinds of data suggest that there is only one PTH gene in the human haploid genome. First, the blots of enzyme digests of uncloned DNA contain only bands predicted by the maps of λ hPTH1 and λ hPTH2. These blots contain fragments generated by several restriction enzymes and probes covering both the far 5' and 3' ends of the gene. These results strongly suggest that the human genome contains only one PTH gene, but they cannot eliminate the possibility that the DNA sequences flanking multiple human PTH genes are tightly conserved in the genome, resulting in identical physical maps of the multiple PTH genes. This possibility is eliminated by the second, quantitative, experiment, in which we measured in uncloned DNA the amount of DNA recognized by PTH-specific probes. By comparing the intensity of autoradiographic bands in DNA blots of uncloned genomic DNA and known amounts of cloned DNA, we could conclude that the genomic bands had the intensities predicted if the PTH gene is a unique gene. In a control well, we mixed together cloned and uncloned DNA and found that the resultant band after blotting had the intensity predicted by simple addition of the intensities associated with each of the DNAs. This result shows that the several micrograms of unrelated DNA in the wells containing uncloned DNA did not interfere with the electrophoresis, blot-transfer, or hybridization of the PTH DNA. Because these blots were hybridized under stringent conditions, we cannot eliminate the possibility that the human genome contains PTH-like genes so different from the PTH gene that they could not be recognized by PTH gene probes.

Potential Z-DNA Structure. The existence of a 14-bp segment of alternating purines and pyrimidines (-3,699 to -3,686) 134 bp upstream from the presumed site of initiation raises many interesting questions regarding the possible role of left-handed Z-DNA in the regulation of human PTH transcription. Negative supercoiling is the major driving force for stabilizing segments of left-handed Z-DNA. Nordheim *et al.* (22) have recently shown that antibody to Z-DNA binds to a 14-bp sequence of alternating purines and pyrimidines with one base pair out of alternation, when the sequence in the plasmid pBR322 is negatively supercoiled in the physiological range. The alternating sequence found near human PTH is 14 bp in length; however, it is part of an 18-bp segment of alternating purines and pyrimidines with one base pair out of alternation. It is thus longer than the segment that has been observed to form Z-DNA in the plasmid pBR322. The sequence near the PTH gene is largely of the type (dC-dA)_n(dG-dT)_n, and DNA segments of this type have been found to form Z-DNA at levels of negative supercoiling that are within the physiological range (23). Hamada *et al.* (24) have shown that longer sequences of the type (dC-dA)_n(dG-dT)_n are widely dispersed through the eukaryotic genome in a number of species. In their assay they determined that there were over 50,000 copies of this type, 50 bp or greater in length, in the human genome.

Recently a number of proteins have been found in the nuclei of *Drosophila* cells that have the ability to bind specifically to left-handed Z-DNA but not to right-handed B-DNA (25). These proteins were found to have the property of stabilizing the Z-DNA conformation, and in particular were shown to bind to negatively supercoiled plasmids containing the sequence (dC-dA)_n(dG-dT)_n. It is reasonable to believe that similar Z-DNA binding proteins exist in human cells. These proteins may be able to stabilize the Z-DNA conformation in the nucleotide sequence 134 bp from the presumed site of transcription initiation of the PTH gene. At the present time we do not know what the effects would be of having Z-DNA binding protein attached to a segment of Z-DNA at this site. If we postulate that the Z-DNA binding proteins bind tightly to this site, and if this site is in the promoter region, the proteins could block the attachment of RNA polymerase and decrease the level of transcription. Alternatively, if such proteins were readily released from this Z-DNA binding site and the local region of Z-DNA converted to one of B-DNA, the release would enhance the negative superhelical density in that region, which, in turn, might promote the level of RNA polymerase binding. Further experimentation is necessary to show whether this region on the 5' side of the PTH gene plays any role in the regulation of gene expression.

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1. Kronenberg, H. M., McDevitt, B. E., Majzoub, J. A., Nathans, J., Sharp, P. A., Potts, J. T., Jr., & Rich, A. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4981-4985.
2. Weaver, C. A., Gordon, D. F. & Kemper, B. (1981) *Proc. Natl. Acad. Sci. USA* 78, 4073-4077.
3. Weaver, C. A., Gordon, D. F. & Kemper, B. (1982) *Mol. Cell. Endocrinol.* 28, 411-424.
4. Hendy, G. N., Kronenberg, H. M., Potts, J. T., Jr., & Rich, A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7365-7369.
5. Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G. & Maniatis, T. (1981) *Cell* 15, 1157-1174.
6. Benton, W. D. & Davis, R. W. (1977) *Science* 196, 180-182.
7. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* 113, 237-251.
8. Seed, B. (1983) *Nucleic Acids Res.*, in press.
9. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* 7, 1513-1523.
10. Southern, E. M. (1975) *J. Mol. Biol.* 98, 503-517.
11. Vogelstein, B. & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA* 76, 615-619.
12. Maxam, A. & Gilbert, W. (1980) *Methods Enzymol.* 65, 499-560.
13. Gross-Bellard, M., Oudet, P. & Chambon, P. (1973) *Eur. J. Biochem.* 36, 32-38.
14. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* 76, 3683-3687.
15. Lewin, B. (1980) *Gene Expression* (Wiley, New York), 2nd Ed., Vol. 2, p. 962.
16. Keutmann, H. T., Sauer, M. M., Hendy, G. N., O'Riordan, J. L. H. & Potts, J. T., Jr. (1978) *Biochemistry* 17, 5723-5729.
17. Breathnach, R. & Chambon, P. (1981) *Annu. Rev. Biochem.* 50, 349-383.
18. Gilbert, W. (1978) *Nature (London)* 271, 501.
19. Young, R. A., Hagenbuehle, O. & Schibler, U. (1981) *Cell* 23, 451-458.
20. Potts, J. T., Jr., Kronenberg, H. M. & Rosenblatt, M. (1982) *Adv. Protein Chem.* 35, 323-396.
21. Cohn, D. V. & MacGregor, R. R. (1981) *Endocrinol. Rev.* 2, 1-6.
22. Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C., Stollar, B. D. & Rich, A. (1982) *Cell* 31, 309-318.
23. Nordheim, A. & Rich, A. (1983) *Proc. Natl. Acad. Sci. USA* 80, 1821-1825.
24. Hamada, H., Petrino, M. G. & Kakunaga, T. (1982) *Proc. Natl. Acad. Sci. USA* 79, 6465-6469.
25. Nordheim, A., Tesser, P., Azorin, F., Kwon, Y. H., Möller, A. & Rich, A. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7729-7733.

Efficient Secretion and Processing of Heterologous Proteins in *Saccharomyces cerevisiae* Is Mediated Solely by the Pre-Segment of α -Factor Precursor

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ABSTRACT

A novel processing site was identified in fusions of the α -factor precursor of *Saccharomyces cerevisiae* following its 19 amino-terminal residues (pre-segment). Fusions of the pre-segment to heterologous proteins, including aminoglycoside phosphotransferase (APH) and human granulocyte-macrophage colony stimulating factor (hGM-CSF), were as efficiently secreted and processed as corresponding pre-pro fusions. Pre- and pre-pro fusions to hGM-CSF were identically *N*- and *O*-glycosylated. While pre-pro fusions to interleukin-1 β were not cleaved, pre-fusions were correctly processed during secretion. The high secretion efficiency of pre-fusions suggests that the pro-segment of the α -factor precursor is not required for efficient secretion and processing of protein fusions.

INTRODUCTION

THE SECRETION PRECURSOR encoded by *MF α 1* in *Saccharomyces cerevisiae* contains an unusually long leader region of 85 amino acids, followed by four tandem repeats consisting of 6-8 residues of a spacer region and the 13 amino acids of α -factor (Kurjan and Herskowitz, 1982). Despite the signal sequence-like characteristics of its 19 amino-terminal residues (pre-segment), no direct evidence exists for processing of the leader (Julius *et al.*, 1984b; Rothblatt and Meyer, 1986). The spacer region, however, is cleaved following a Lys-Arg sequence and at each of the subsequent Glu/Asp-Ala sequences by the *KEX2* and *STE13* gene products, respectively (Julius *et al.*, 1983, 1984a). Fusions of the entire leader region (pre-pro region) to many heterologous proteins are secreted very efficiently and correctly processed at *KEX2* and *STE13* sites following the pro-segment (Bitter *et al.*, 1984; Brake *et al.*, 1984; Singh *et al.*, 1984; Ernst, 1986). Thus, the pro-region might contain positive signals required for effective secretion (sorting) into the growth medium. Alternatively, the particular efficiency of α -factor pre-pro fusions might be

due solely to its pre-segment. Pre-segment cleavage could conceivably be affecting secretion by influencing the release of the secretion precursor from membranes of the endoplasmic reticulum.

Some indirect evidence suggests that cleavage of the pre-segment can occur in the α -factor precursor and in pre-pro fusions to proteins. A proteolytic fusion fragment starting with residue 20 of α -factor precursor was detected in the culture fluid of a yeast strain expressing a pre-pro/hGM-CSF fusion (Ernst *et al.*, 1987). In addition, α -factor precursor and a pre-segment fusion are truncated during secretion *in vivo* and *in vitro* (Rothblatt *et al.*, 1987; Sidhu and Bollon, 1987), consistent with pre-segment cleavage. The present study analyzes fusions of the 19, 22, and 85 amino-terminal residues of the α -factor precursor to three heterologous reporter proteins. The results define the pre-segment by demonstrating that only the 19 amino-terminal residues are required for effective secretion and processing. There was no evidence for an effect of the pro-region on secretion and processing. These findings suggest a novel, efficient way for secretion of homologous and heterologous proteins in yeast.

MATERIALS AND METHODS

Host strain and growth conditions

Yeast host for recombinant vectors was *S. cerevisiae* BJ1991 (*MAT α ura3-52 leu2-3,112 trp1 prb1-1122 pep4-3*) (Ernst, 1986). Transformants were grown in minimal medium supplemented with 4% casamino acids, as described previously (Ernst, 1986).

Plasmids

Pre-pro fusions were constructed as described previously (Ernst, 1986), such that the first amino acid of the heterologous protein was joined to the Lys-Arg sequence constituting the processing site for the *KEX2* gene product (Julius *et al.*, 1984a). The occurrence of a convenient *Hinc* II site in *MF α 1* close to the region corresponding to the pre-segment cleavage point allowed fusions of a pre-segment elongated by 3 amino acids (Ala-Pro-Val) to heterologous proteins (pre*-fusions). The extra residues were removed in later constructions using primer mutagenesis (Oostra *et al.*, 1983) by placing a *Nae* I site (GCC GGC) into the *MF α 1* region corresponding to the pre-cleavage site (Ala19-Ala20). Thus, cleavage with *Bam* HI and *Hinc* II (or *Nae* I) of pER562 (Ernst *et al.*, 1987) (or its *Nae* I derivative) yielded a fragment encoding the *ACT* (actin) promoter joined to the pre* (or pre-) segment, which was ligated with a blunt-end/*Hind* III fragment encoding the heterologous protein and inserted between the *Bam* HI and *Hind* III sites of JDB207 (Beggs, 1981). For aminoglycoside phosphotransferase (APH) constructions a 1.3-kb *Xho* I-*Sal* I fragment of Tn903 was excised from pEX-4 (Oka *et al.*, 1981; Ernst and Chan, 1985) and inserted into the *Sal* I site of pUC8 such that the *Xho* I-*Sal* I junction was distal of the pUC8 *Hind* III site. This plasmid was cut with *Bam* HI, filled in using Klenow polymerase I, and recut with *Hind* III. The resulting fragment encoding a Δ 10 APH protein commencing with Asp-Pro residues derived from pUC8 was joined to pre*- and pre-pro sequences as outlined above. Pre- (pER873) and pre-pro- (pER562) fusions to human granulocyte-macrophage colony stimulating factor (hGM-CSF) were constructed using a 0.4-kb *Nco* I (S₁-treated)-*Hind* III fragment of p210* (Ernst *et al.*, 1986) carrying the coding region for mature hGM-CSF. To construct pre- and pre-pro fusions to human interleukin-1 β (IL-1 β) a 0.5-kb *Nco* I (S₁)-*Hind* III fragment encoding mature IL-1 β (Wingfield *et al.*, 1986) was used. Vector pEX-4 has been described (Ernst and Chan, 1985).

Protein procedures

NaDodSO₄-polyacrylamide gel electrophoresis (15% gel) and immunoblotting procedures were carried out on cultures of yeast transformants grown to the beginning of the stationary growth phase, as described previously (Ernst *et al.*, 1987; Moonen *et al.*, 1987). For all proteins, qualitatively similar results were obtained with transformants

grown to the logarithmic growth phase (data not shown). Conditions for endoglycosidase H (EndoH) treatment have been described (Ernst *et al.*, 1987). Rabbit polyclonal antibodies against APH, hGM-CSF, and IL-1 β expressed in *E. coli* were kindly supplied by M. Hirschi, Biogen S.A. For amino-terminal analysis the IL-1 β bands were isolated by preparative NaDodSO₄-polyacrylamide gel electrophoresis and sequenced as described (Ernst *et al.*, 1987).

RESULTS

Secretion vectors

Secretion vectors for *S. cerevisiae* were constructed that encode proteins containing the 19, 22, and 85 amino-terminal residues of α -factor precursor (pre-, pre*, and pre-pro segments) fused to the coding region of heterologous proteins. To probe different aspects of yeast secretion the following three proteins were chosen: a cytoplasmic protein (APH), a N- and O-glycosylated protein (hGM-CSF), and a protein that is secreted as part of an extensive secretion precursor (IL-1 β). Protein fusions were encoded on expression vectors containing *LEU2* and 2 μ sequences for selection and replication in yeast; signals for initiation and termination of transcription of gene fusions were provided by the *ACT* promoter and the *FLP* terminator (Beggs, 1981; Ernst, 1986).

Secretion of heterologous proteins

To determine the effect of the pre-segment on secretion efficiency pre- and pre-pro fusions to the cytoplasmic protein APH were examined. APH is a 26-kD protein encoded by Tn903 that directs G418-(kanamycin) resistance in yeast (Ernst and Chan, 1985). Fusions to a cytoplasmic protein were studied because regions within the protein required for secretion, or sorting (Schauer *et al.*, 1985; Haguenaer-Tsapis *et al.*, 1986) should be absent; APH lacks Asn-X-Ser/Thr sequences that could become N-glycosylated and thereby affect secretion efficiencies (Ernst *et al.*, 1987). Culture fluids of yeast strains expressing pre* (pER711) and pre-pro (pER678) fusions contained equal amounts of the 26-kD APH protein, indicating equally efficient cleavage of pre- and pre-pro regions (Fig. 1). Both transformant strains grew equally well as a nontransformed control strain and showed no signs of lysis, as determined by microscopic inspection and by analysis of medium proteins by NaDodSO₄-polyacrylamide gel electrophoresis (also note in Fig. 1 the absence of cross-reacting intracellular proteins in the medium). In addition, APH expressed by vector pEX-4 was detected only intracellularly (Fig. 1). Therefore, the presence of APH in the culture fluid was due to secretion and not cell lysis. With both secretion vectors, pER711 and pER678, about 5% of total APH (0.2–0.5 mg/liter) were secreted (OD_{600 nm} = 10). In association with cells, the same 26 kD protein, as well as smaller amounts of a second unidentified protein (35 kD for pER678; 45 kD for pER711) were detected by the APH

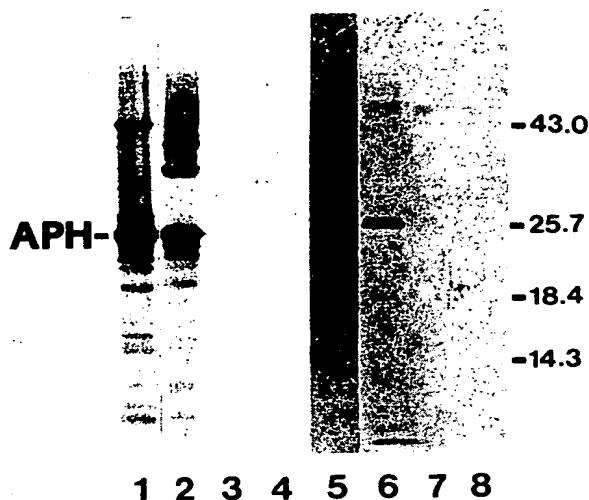


FIG. 1. Secretion of pre*- and pre-pro-APH fusions. Cultures of yeast transformants were centrifuged and 30 μ l of culture medium concentrated tenfold (lanes 5–8), or cells contained in 30- μ l culture medium (lanes 1–4) were subjected to NaDodSO₄-polyacrylamide gel electrophoresis, followed by immunoblotting using rabbit anti-APH. Lanes 1 and 5, pre*-fusion (pER711); lanes 2 and 6, pre-pro fusion (pER678); lanes 3 and 7, intracellular expression plasmid pEX-4; lanes 4 and 8, negative control vector JDB207. Numbers on the right side of the figure indicate the migration of (BRL) molecular weight standards (kD).

antibody. Thus, the presence of the pro-segment in fusions to a cytoplasmic protein did not have any effect on secretion.

To explore the effect of the pro-region on glycosylation, pre- and pre-pro fusions to hGM-CSF that become extensively *N*- and *O*-glycosylated during secretion by yeast were studied (Ernst *et al.*, 1987). Besides the 14.5-kD unglycosylated protein, *N*-glycosylated derivatives of 18 kD and about 50 kD and *O*-glycosylated forms of 15.5 kD, 19 kD, and about 50 kD are secreted. *N*- and *O*-glycosylation in yeast occurs after the nascent protein chain has traversed the membrane of the endoplasmic reticulum (Haselbeck and Tanner, 1983). If the pro-region prolonged the duration the precursor remains attached to the membrane, for example by inhibition of processing of the pre-segment, more extensive *N*- and *O*-glycosylation might result. Quantity (about 20 mg/liter at OD₆₀₀ = 10) and quality of cell-associated and secreted hGM-CSF derived from pre- and pre-pro fusions were similar (Fig. 2). Thus, the identical secretion and glycosylation obtained with pre- and pre-pro fusions does not indicate that the pro-region has any effect on the secretion pathway.

Fusions of the pre-pro segment to heterologous proteins are occasionally not cleaved by the *KEX2* processing enzyme (Thim *et al.*, 1986). Pre-pro fusions to IL-1 β were not processed, resulting in secretion of very small amounts of a highly glycosylated secretion precursor (data not

shown). Both pre*- (pER822) and pre- (pER870) fusions were effectively secreted and processed (about 10 mg/liter secreted at OD_{600 nm} = 10) (Fig. 3). The secreted IL-1 β was partially *N*-glycosylated, resulting in a 17-kD unglycosylated and a 20-kD glycosylated protein that was reduced in size to 17 kD by EndoH treatment (Fig. 3). Interestingly, a third protein of 18.5 kD reactive with anti-IL-1 β was detected in association with cells, consistent with lack of pre-cleavage of this protein. It should be noted that the first residues of IL-1 β Ala-Pro-Val are identical to the amino acids following the pre-cleavage point in α -factor precursor. This sequence may contribute to delayed processing of the pre-segment.

Protein analyses

The 20-kD form of secreted IL-1 β was isolated by preparative NaDodSO₄-polyacrylamide gel electrophoresis and subjected to automatic Edman degradation (Table 1). With pre*-fusions, an IL-1 β variant elongated by three amino acids derived from the *MF α 1* region was secreted, indicating that the α -factor precursor pre-segment was cleaved following residue 19. In contrast, a pre-fusion was processed correctly during secretion (Table 1).

DISCUSSION

These findings provide experimental evidence that the 19 amino-terminal residues of the α -factor precursor (pre-segment) are sufficient to mediate efficient secretion and processing of heterologous proteins in *S. cerevisiae*. This result has implications regarding the role of pre- and pre-pro segments of α -factor precursor in secretion and also demonstrate an efficient alternative for secretion of homologous, or heterologous proteins in yeast.

It has previously been suggested that the structural portions of secreted yeast proteins contain signals important for secretion efficiency or for sorting (Schauer *et al.*, 1985; Haguenaer-Tsapis *et al.*, 1986). Conceivably, the pro-segment of the yeast α -factor precursor (Kurjan and Herskowitz, 1982) could contain such signals. The present work shows that pre-fusions to three unrelated heterologous proteins are secreted and that pre- and pre-pro fusions have equal secretion efficiencies. At least two hypotheses are compatible with these results. First, the efficient secretion of pre-pro fusions could be due solely to the pre-segment. In this case, neither the pro-sequence nor the chosen heterologous proteins are assumed to contain sequences stimulating secretion. Second, the pro-segment, as well as the heterologous proteins tested might contain sequences stimulating secretion. Several assumptions have to be made for this hypothesis, including the presence of fortuitous stimulatory sequences in a cytoplasmic protein (APH) and of fortuitous, or natural stimulatory sequences in two human proteins that are also efficiently recognized in a heterologous host. Thus, in the absence of definitive proof for a secretion enhancer sequence in other systems, the simplest ex-

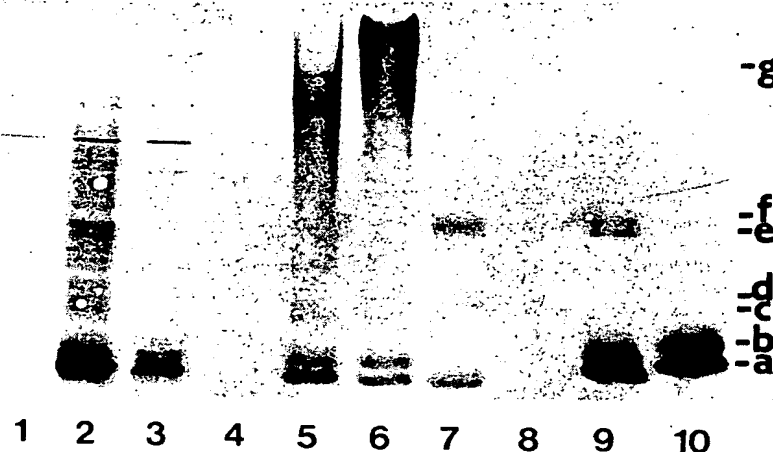


FIG. 2. Secretion of pre- and pre-pro-hGM-CSF fusions. Cultures of yeast transformants were centrifuged and cells contained in 30 μ l of medium (lanes 1–3), or 30 μ l of medium (lanes 4–6 and 8–10) were analyzed by NaDodSO₄-polyacrylamide gel electrophoresis, followed by immunoblotting using rabbit anti-hGM-CSF. Lanes 1 and 4, Control vector JDB207; lanes 2 and 5, pre-pro fusion (pER562); lanes 3 and 6, pre-fusion (pER873); lane 7, standard proteins (BRL) (the 14.3 kD and 25.6 kD standards cross-react with anti-hGM-CSF); lane 8, 30 μ l of medium of the pJDB207 transformant was treated with EndoH; lane 9, as lane 8, but with medium of the pER562 transformant; lane 10, as lane 8, but with medium of the pER873 transformant. The positions of hGM-CSF forms (Ernst *et al.*, 1987) are denoted by letters: a, 14.5 kD (unglycosylated); b, 15.5 kD (*O*-glycosylated); c, 18 kD core-*N*-glycosylated; d, 19 kD, core and *O*-glycosylated; e, 25 kD deglycosylated precursor; f, *O*-glycosylated precursor; g, heterogeneous *N*-glycosylated 50-kD form.

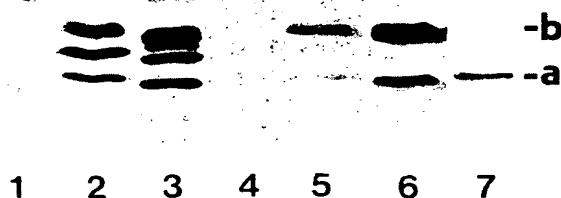


FIG. 3. Secretion of pre- and pre*-IL-1 β fusions. Cell-bound (lanes 1–3) and medium IL-1 β (lanes 4–7) expressed by yeast transformants was analyzed by NaDodSO₄-polyacrylamide gel electrophoresis, followed by immunoblotting using rabbit anti-IL-1 β as described in the legend for Fig. 2. Lanes 1 and 4, Control vector JDB207; lanes 2 and 5, pre*-fusion (pER822); lanes 3 and 6, pre-fusion (pER870); lane 7, 30 μ l of medium of the pER870 transformant treated with EndoH. a, 17-kD form; b, 20-kD form.

planation, *i.e.*, the absence of positive secretion signals within the pro-region of α -factor precursor, appears most likely. We previously discovered that the degree of protein glycosylation can affect the efficiency of secretion (Ernst *et al.*, 1987). Because pre- and pre-pro fusions to a unglycosylated protein are equally secreted the glycosylation of the pro-segment appears not to be a significant factor in secretion. A positive biological role for the pro-segment is however suggested by the inability of *mfal mfal* double mutants, which do not express α -factor precursor, to mate in the presence of exogenously supplied α -factor (Kurjan, 1986).

Our previous finding of pre-segment processing *in vivo* (Ernst *et al.*, 1987) shows that the presence of the pro-segment does not prevent pre-segment processing. This observation is particularly important since processing of signal sequences *in vivo* and *in vitro* is not necessarily identical (Rottier *et al.*, 1987). Residues following the pre-cleavage site may nevertheless contribute in modifying the velocity of the processing step (Haguenauer-Tsapis *et al.*, 1986). In agreement with this notion, a pre-fusion to IL-1 β , which commences with identical residues as the pro-region itself (Ala-Pro-Val) appeared to be processed incompletely. The uncleaved protein species was found exclusively associated with the cell and no uncleaved glycosylated protein (expected size about 21.5 kD) was found, suggesting that signal cleavage is required for effective secretion and *N*-glycosylation (Haguenauer-Tsapis *et al.*, 1986). Delayed processing of the pre-region may be the reason why pre-segment processing was not observed initially (Julius *et al.*,

HETEROLOGOUS PROTEIN SECRETION IN YEAST

TABLE 1. AMINO-TERMINAL RESIDUES OF IL-1 β SECRETED BY *S. cerevisiae*^a

Plasmid	Protein	Sequence													
pER822	Pre*-fusion	15	16	17	18	19	20	21	22						
	secreted protein	<u>S</u>	<u>S</u>	<u>A</u>	<u>L</u>	<u>A</u>	<u>A</u>	<u>P</u>	<u>V</u>	A	P	V	R	S	E
pER870	Pre-fusion	15	16	17	18	19									
	secreted protein	<u>S</u>	<u>S</u>	<u>A</u>	<u>L</u>	<u>A</u>	A	P	V	R	S	L	N		

^aResidues derived from the α -factor-precursor are underlined and numbered.

1984b; Rothblatt *et al.*, 1986). Recent results, however, suggest that the pre-segment processing observed in pre-pro fusions to heterologous proteins can also occur in the α -factor precursor itself (Rothblatt *et al.*, 1987).

The results demonstrate that pre-fusions to heterologous proteins are an effective alternative to conventional pre-pro α -factor fusions (Bitter *et al.*, 1984; Brake *et al.*, 1984; Singh *et al.*, 1984; Ernst, 1986). Because of the potentially inhibitory effects of residues following the pre-segment on its processing, precise fusions of the pre-segment to the heterologous protein should be most effective. As demonstrated with IL-1 β fusions, correct pre-cleavage can even occur in cases where corresponding pre-pro fusions are not processed at the KEX2 site.

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REFERENCES

- BEGGS, J.D. (1981). Multiple-copy yeast vectors. In *Molecular Genetics in Yeast*, Alfred Benzon Symposium 16, D. von Wettstein, J. Fries, M. Kielland-Brand, and A. Staderup, Eds. (Munksgaard, Copenhagen) pp. 383-389.
- BITTER, G.A., CHEN, K.K., BANKS, A.R., and LAI, P.-H. (1984). Secretion of foreign proteins from *Saccharomyces cerevisiae* directed by α -factor gene fusions. *Proc. Natl. Acad. Sci. USA* **81**, 5330-5334.
- BRAKE, A.J., MERRYWEATHER, J.P., COIT, D.G., HEBERLEIN, U.A., MASIARZ, F.R., MULLENBACH, G.T., URDEA, M.S., VALENZUELA, P., and BARR, P.J. (1984). α -Factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **81**, 4642-4646.
- ERNST, J.F., and CHAN, R.C. (1985). Characterization of *Saccharomyces cerevisiae* mutants supersensitive to aminoglycoside antibiotics. *J. Bacteriol.* **163**, 8-14.
- ERNST, J.F. (1986). Improved secretion of heterologous proteins by *Saccharomyces cerevisiae*: Effects of promoter substitution in alpha factor fusions. *DNA* **5**, 483-491.
- ERNST, J.F., MERMOD, J.-J., DELAMARTER, J.D., MAT-TALIANO, R., and MOONEN, P. (1987). O-Glycosylation and novel processing events during secretion of α -factor/GM-CSF fusions in *S. cerevisiae*. *Bio/Technology* **5**, 831-834.
- HAGUENAUER-TSAPIS, R., NAGY, M., and RYTER, A. (1986). A deletion that includes the segment coding for the signal peptidase cleavage site delays release of *Saccharomyces cerevisiae* acid phosphatase from the endoplasmic reticulum. *Mol. Cell. Biol.* **6**, 723-729.
- HASELBECK, A., and TANNER, W. (1983). O-Glycosylation in *Saccharomyces cerevisiae* is initiated at the endoplasmic reticulum. *FEBS Lett.* **158**, 335-338.
- JULIUS, D., BLAIR, L., BRAKE, A., SPRAGUE, G., and THORNER, J. (1983). Yeast α -factor is processed from a larger precursor polypeptide: The essential role of a membrane-bound dipeptidyl aminopeptidase. *Cell* **32**, 839-852.
- JULIUS, D., BRAKE, A., BLAIR, L., KUNISAWA, R., and THORNER, J. (1984a). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro- α -factor. *Cell* **37**, 1075-1089.
- JULIUS, D., SCHEKMAN, R., and THORNER, J. (1984b). Glycosylation and processing of prepro- α -factor through the yeast secretory pathway. *Cell* **36**, 309-318.
- KURJAN, J., and HERSKOWITZ, I. (1982). Structure of a yeast pheromone gene (*MF α*): A putative α -factor precursor contains four tandem copies of mature α -factor. *Cell* **30**, 933-943.
- KURJAN, J. (1985). α -Factor structural gene mutations in *Saccharomyces cerevisiae*: Effects on α -factor production and mating. *Mol. Cell. Biol.* **5**, 787-796.
- MANIATIS, T., FRITSCH, E.F., and SAMBROOK, J. (1982). In *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
- MOONEN, P., MERMOD, J.-J., ERNST, J.F., HIRSCHI, M., and DELAMARTER, J.F. (1987). Increased biological activity of deglycosylated recombinant human granulocyte/macrophage colony-stimulating factor produced by yeast or animal cells. *Proc. Natl. Acad. Sci. USA* **84**, 4428-4431.
- OKA, A., SUGISAKI, H., and TAKANAMI, M. (1981). Nucleotide sequence of the kanamycin resistance transposon Tn903. *J. Mol. Biol.* **147**, 217-226.

- OOSTRA, B.A., HARVEY, R., ELY, B.K., MARKHAM, A.F., and SMITH, A. (1983). Transforming activity of polyoma virus middle-T antigen probed by site-directed mutagenesis. *Nature* **304**, 456-459.
- ROTHBLATT, J.A., and MEYER, D.I. (1986). Secretion in yeast: Reconstitution of the translocation and glycosylation of α -factor and invertase in a homologous cell-free system. *Cell* **44**, 619-628.
- ROTHBLATT, J.A., WEBB, J.R., AMMERER, G., and MEYER, D.I. (1987). Secretion in yeast: Structural features influencing the posttranslational translocation of prepro- α -factor *in vitro*. *EMBO J.* **6**, 3455-3463.
- ROTTIER, P.J.M., FLORKIEWICZ, A.S., SHAW, A.S., and ROSE, J.K. (1987). An internalized amino-terminal signal sequence retains full activity *in vivo* but not *in vitro*. *J. Biol. Chem.* **262**, 8889-8895.
- SCHAUER, I., EMR, S., GROSS, C., and SCHEKMAN, R. (1985). Invertase signal and mature sequence mutants that delay intercompartmental transport of active enzyme. *J. Cell. Biol.* **100**, 1664-1675.
- SIDHU, R.S., and BOLLON, A.P. (1987). Analysis of α -factor secretion signals by fusing with acid phosphatase of yeast. *Gene* **54**, 175-184.
- SINGH, A., LUGOVOY, J.M., KOHR, W.J., and PERRY, L.J. (1984). Synthesis, secretion and processing of α -factor-interferon fusion proteins in yeast. *Nucleic Acids Res.* **12**, 8927-8938.
- THIM, L., HANSEN, M.T., NORRIS, K., HOEGH, L., BOEL, E., FORSTROM, J., AMMERER, G., and FILL, N. (1986). Secretion and processing of insulin precursors in yeast. *Proc. Natl. Acad. Sci. USA* **83**, 6766-6770.
- WINGFIELD, P., PAYTON, M., TAVERNIER, J., BARNES, M., SHAW, A., ROSE, K., SIMONA, M.G., DEMSZID, WILLIAMSON, K., and DAYER, J.-M. (1986). Purification and characterization of human interleukin-1 β expressed in recombinant *Escherichia coli*. *Eur. J. Biochem.* **160**, 491-497.

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(54) Process for expressing heterologous protein in yeast, expression vehicles and yeast organisms therefor.

(57) The isolation of the yeast α -factor genes is described. The promoter and signal peptide portions are isolated and joined to DNA coding for proteins heterologous to yeast in a plasmid which is used to transform yeast cells. The yeast expresses the heterologous DNA and processes and secretes the heterologous protein.

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Docket No. 100/175

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PROCESS FOR EXPRESSING HETEROLOGOUS PROTEIN IN
YEAST, EXPRESSION VEHICLES AND YEAST ORGANISMS
THEREFOR

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This application is related to US applications Serial No.
06/438,236 (EP 88632), and Serial No. 06/488,337 filed 25 April 1983
(European application filed on even date), the disclosures of
which are hereby incorporated by reference.

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Field of the Invention

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This invention is directed generally to recombinant DNA
technology utilizing yeast host systems and expression vehicles that
produce, process and secrete heterologous protein as discrete

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product unaccompanied by interfering amounts of unwanted presequence or other artifact of expression.

Proteins that are secreted through the cell membrane of the parent cell are ordinarily produced in the cell as a "pre"-protein. In that form, the protein is fused to an additional polypeptide sequence which presumably assists its secretion and localization. This additional protein, referred to as a "signal" polypeptide, is believed to be clipped from the secreted "mature" protein during the secretion process. Although the signal peptides of pre-proteins share some similarities, their primary structures differ considerably. The signal peptides even for a given organism exhibit this variation. For example, the signal for human growth hormone is substantially different from the signal for human insulin. This suggests that each protein has evolved with a signal sequence which is particularly well suited for translocation of that particular protein through a cell membrane.

This invention is based upon the discovery that a substantially mature protein is produced and often secreted by yeast when the DNA coding for the heterologous protein is operably attached to the DNA sequence of the promoter and/or signal peptide encoding portions of the yeast α -factor gene. (It will be apparent from the disclosure herein that yeast harbors at least two alpha factor genes. The use of "the alpha factor gene" is intended to include all such functional genes.) Thus, in a primary aspect, this invention is directed to the means and methods of obtaining useful quantities of heterologous protein from the medium of a yeast culture containing viable cells harboring expression vehicles containing DNA encoding the desired protein, wherein the DNA coding for this heterologous protein is operably connected to a DNA sequence comprising the promoter and/or signal portion of the yeast α -factor gene. Of enormous advantage is the enablement, by this invention, of obtaining useful, discrete protein product in the cell culture medium by expression of heterologous DNA in an easily modified plasmid.

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5 The alpha factor of yeast contains a "pre-pro" sequence which is
ordinarily removed from the α -factor upon the completed act of
secretion. Operationally, therefore, the pre-pro sequence functions
as a signal sequence in the process of secretion into the medium as
will be further explained below. It is clear from the results
10 obtained herein that the peptide which comprises the thus-defined
signal sequence of alpha factor fused to a heterologous protein is
successfully processed by the yeast organism so as to result in the
secretion of the mature heterologous protein into the surrounding
medium. Therefore, the advantages obtained by use of this "pre-pro"
15 signal are realized whether or not the expression of the
signal/heterologous protein gene sequence is under the control of
the alpha factor promoter or under the control of other promoters
which are functional in yeast. Similarly, the results obtained
demonstrate that the alpha factor promoter is effective in
20 expressing the heterologous gene, and that such expression could be
obtained without the intermediate insertion of the signal sequence
into the expression vehicle. Accordingly, this invention is
directed to the use of alpha factor promoter qua promoter in yeast
systems for the expression of heterologous peptides and to the use
of the alpha factor signal qua signal as a means for effecting
processing and secretion of heterologous proteins produced as a
result of expression in yeast.

25 The publications and other materials referred to herein to
illuminate the background of the invention, and in particular cases,
to provide additional detail respecting its practice, are
incorporated herein by reference, and for convenience, are
numerically referenced and grouped in the appended bibliography.

30 Background of the Invention

35 Yeast organisms naturally transport a small number of certain
homologous proteins to, and sometimes through, the plasma membrane

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as an essential contribution to cell surface growth and cell metabolism. As the cell buds as an incident of reproduction preparatory to formation of a daughter cell, additional proteins are required for formation of cell wall and plasma membrane as well as for metabolism. Some of these proteins must find their way to the site of function; hence, a secretory pathway is believed to exist (1). Certain homologous proteins involved in the above processes are formed by translation by ribosomes attached to the endoplasmic reticulum. Homologous proteins are those normally produced by the yeast species and required for its viability. Once formed, they migrate by transfer to Golgi apparatus, thence within vesicles to plasma membranes where some associate, or to some extent, penetrate into the space between the plasma membrane and the cell wall. A small number of homologous proteins seems to be exported completely through the cell wall, such as α -factor and killer toxin (2,3).

Again, the bud region of the cell seems to be the site of attraction for the vesicles and by their fusion to the inner surface of the bud they contribute to the overall growth of the plasma membrane, and presumably, the cell wall (4,5,6). It is controversial still whether glycosylation of the protein may assist, or is implicated, in the so-called secretory process. Further, by definition "secreted" proteins are believed to have a signal prepeptide, postulated to be associated with the transport or incorporation process at the membrane surface. However, the precise mechanism involved in the overall secretory process is not fully understood.

It was contemplated that recombinant DNA technology could provide valuable assistance in answering the open questions about the secretory process in yeast organisms and, given its proven applicability in enabling such, and other, organisms to produce copious quantities of heterologous polypeptide products endogenously (See, e.g., 7 to 17), in achieving appropriate manipulation of the yeast host so as to direct the secretion of heterologous protein in

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discrete, mature form. This has, in fact, been achieved and is the subject of US 438,236 (EP 88632),

supra. In that application is described the discovery that a heterologous protein, initially expressed as a pre-protein with its native signal or hybrid thereof, can be processed and secreted by yeast as a mature protein.

Summary of the Invention

This invention is based on the discovery that yeast organisms can be caused to produce, process and secrete protein that is normally heterologous to the yeast organism and not required for its viability, such that the protein can be obtained from the medium supporting the viable and reproducing yeast cells and in discrete form substantially unaccompanied by unwanted peptide presequence or other artifact of expression. For this purpose, a DNA sequence encoding the desired, heterologous protein is linked to the DNA sequence encoding the non-native (to the protein) signal sequence of yeast α -factor. Suitable yeast cells are transformed with expression vehicles harboring such DNA encoding a heterologous protein operably connected to the such DNA coding for the α -factor signal (pre-pro) peptide and a promoter. Upon expression of the sequence encoding the heterologous protein together with that encoding α -factor signal peptide, the expression product is processed and the mature heterologous protein is exported into the medium of the cell culture, from which it can be removed with relative ease, without need to disrupt the viable yeast cells. It is thus recovered in otherwise substantially mature form for use, without the need to remove unwanted presequence or certain other artifacts of expression (e.g., the methionine attached to the otherwise first N-terminus amino acid which is an expressional consequence of the AUG translational start signal codon). Thus, the medium can be obtained in a form substantially free of viable or disrupted (i.e., lysed or otherwise broken) cells and, since it contains the desired product, is susceptible to more easily employed

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purification techniques. Such product, after purification, is fit for use as intended. For example, human leukocyte interferon product finds use as a human antiviral and/or antitumor agent (See, generally, 7 to 17).

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In summary, the present invention comprises the use of yeast alpha factor signal sequences and/or promoter to produce a protein normally heterologous to a yeast organism and not required for its viability, in discrete form unaccompanied by any substantial peptide presequence or other artifact of expression, as a product of yeast expression, processing and secretion. Further, this invention provides yeast cultures capable of producing such protein and resultant yeast culture media containing such protein as product. More specifically, the invention is directed to a process for producing heterologous proteins in yeast, and the expression vehicles and organisms employed in this process, wherein the alpha factor promoter is used to effect the expression of the foreign gene. Further, the invention is directed to the use of the signal (pre-pro) sequence for alpha factor to effect the processing and secretion of an expressed foreign protein, to a recombinant expression vehicle effectively harboring the alpha factor DNA sequences and to the cells transformed with such vehicles.

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By the term "heterologous protein" as used herein is meant protein that is not normally produced by or required for viability of a yeast organism. This term contemplates the functional insertion of DNA encoding such protein, via recombinant DNA technology, into an expression vehicle, in turn used to transform a yeast organism host. Functional insertion of DNA denotes the insertion of DNA encoding the heterologous protein into an expression vector under control of the α -factor promoter and/or connected to the DNA sequence coding for the α -factor signal to obtain a hybrid preprotein, i.e., one which comprises the α -factor signal peptide fused to the heterologous protein. Examples of such heterologous protein are hormones, e.g., human growth hormone,

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bovine growth hormone, etc.; lymphokines; enzymes; interferons, e.g., human fibroblast, human immune and human and hybrid leukocyte interferons, bovine interferons etc.; viral antigens or immunogens, e.g., foot and mouth disease antigens, influenza antigenic protein, hepatitis core and surface antigens, etc.; factors incidental to growth, e.g. human insulin-like growth factor (IGF-1 and IGF-2), epidermal growth factor (EGF) and nerve growth factor (NGF) and various other polypeptides, e.g., rennin, human serum albumin, human insulin, various glycoproteins, etc.

"Secretion" as used herein means exportation of product through the plasma membrane and at least into or through the cell wall of the yeast organism into the medium supporting the cell culture. In this connection, it will be understood that in some instances, "secreted" product associates in some manner with the cell wall, perhaps necessitating a different purification procedure or a modification of the structure and function of the yeast host.

"Processing" means the cellular cleavage of the α -factor signal peptide from the mature protein so as to produce the heterologous protein unaccompanied by any substantial portion of the signal sequence or by extraneous peptide in--so-called discrete--mature form. By "extraneous" peptide is included peptide artifacts of expression such as methionine. Processing admits of cleavage of the signal polypeptide at a locus inconsequentially removed from the precise point of signal peptide union with mature protein.

Brief Description of the Drawings

Fig. 1 illustrates the structure of pools of synthetic oligonucleotides used as hybridization probes to isolate the gene for α -factor.

Fig. 2 illustrates the results of electrophoresis of DNA

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fragments obtained using the probes of Fig. 1.

Figs. 3 and 4 are the nucleotide sequences of α -factor genes.

5 Fig. 5 illustrates the scheme for joining the gene for human interferon D with the gene for the α -factor promoter and signal sequence.

10 Fig. 6 illustrates the scheme for construction of a yeast expression plasmid for expression of human interferon D (IFN- α_1).

Fig. 7 depicts the protein and DNA sequence at the junction of the α -factor signal sequence and the modified IFN- α_1 gene.

15 Fig. 8 shows the levels of IFN- α_1 in the medium and cell extracts of a culture of a yeast transformant expressing IFN- α_1 .

Fig. 9 illustrates the scheme for construction of a yeast/E. coli shuttle vector for expression of heterologous genes using the α -factor promoter and signal polypeptide gene sequences.

Fig. 10 illustrates the assembly of a yeast/E. coli plasmid for expression of tissue plasminogen activator.

25 Fig. 11 depicts the construction used to effect the production of mature heterologous proteins (human interferon illustrated) as a product of expression with partial alpha factor signal sequence, processing of the alpha factor component and secretion of the mature protein into the supporting medium.

30 Fig. 12 illustrates the degree of consensus between the MF α_1 and MF α_2 polypeptides of figures 3 and 4.

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Detailed Description of a Preferred Embodiment

The yeast Saccharomyces cerevisiae secretes only a limited number of proteins into the culture medium. One of the proteins that is found in the medium is α -pheromone or α -factor (2). Duntze and coworkers (18, 19) first determined that the α -factor is a family of four oligopeptides of 12-13 amino acid residues having the basic sequence

H₂N-(Trp)-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met (or MetSO)-Tyr-COOH.

Figure 3 shows the location of the four peptides in the unprocessed product of one of the α -factor genes, MF α -1. Only the "boxed" segments are secreted into the medium, the remaining sequences are not. It is not clear how much of the remaining sequence is "true" signal (pre) sequence, which is at least partially processed to effect secretion, and how much is "pro" sequence in the sense of a traditional precursor protein (e.g., prorennin, proinsulin.)

Similarly, only the "boxed" portions in product of the MF α 2 gene shown in Figure 4 are secreted, and the nature of the remaining sequences can be described analogously to that of those in MF α 1.

During the reduction of this invention to practice, another group (44) succeeded in isolating and sequencing one of the genes for α -factor (MF α 1) by methods different from those disclosed herein. As described below, two α -factor genes were isolated by us and expression vectors in which the DNA sequence for the promoter and signal peptide of α -factor was inserted in tandem with the DNA sequence coding for heterologous protein were constructed from one of them.

A. Bacterial and Yeast Strains

E. coli K-12 strain 294 (endA thi⁻ hsr⁻ hsm⁺)(ATTC

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31446)(22) was used for bacterial transformations. Yeast strain 20B-12 (α , trp1 pep4) deposited without restriction in the American Type Culture Collection, ATCC No. 20626, on March 5, 1982 was used as yeast host.

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B. Growth Media

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The routine yeast growth medium contained 1 percent Bacto-yeast extract, 2 percent Bacto-peptone and 2 percent dextrose. Yeast minimal medium contained 0.67 percent Bacto-yeast nitrogen base without amino acids, 2 percent dextrose and 3 percent gar. The minimal medium supplemented with 1M sorbitol was used for yeast transformations. Bacterial growth medium was LB (25) which

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was supplemented with 20 μ g/ml ampicillin when used for transformation. S-agar plates used for colony screening contained per liter: 32g tryptone, 5g NaCl, 15g Difco agar and 0.2g NaOH to which ampicillin or chloramphenicol was added as indicated.

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C. Transformations

E. coli 294 was transformed using a published procedure (23). Yeast were transformed essentially as described (21, 24).

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D. Enzymes and DNA Preparations

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Restriction enzymes were purchased from New England Biolabs and Bethesda Research Laboratories and were used according to manufacturer's recommendations. T4 DNA ligase was from New England Biolabs and was used in 20mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP at 14°C. Calf alkaline phosphatase was purchased from Boehringer Mannheim and was used in 100mM NaCl, 50mM Tris-HCl (pH 7.4), 10mM $MgSO_4$, 1mM 2-mercapto-ethanol at 37°C.

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Plasmid DNAs were prepared by the cleared lysate method (29) and were purified by Bio-Rad Agarose A-50 column chromatography. Small amounts of plasmid DNAs from individual E. coli transformants were prepared by a quick-screening procedure (20). DNA restriction fragments were isolated by electroelution from a 1 percent agarose gel followed by phenol/chloroform extraction and ethanol precipitation. Oligo-deoxynucleotide probes were prepared by the phosphotriester method (41).

E. Design of the Hybridization Probe

The 15-mer oligonucleotide probes for the α -factor gene were designed on the basis of the amino acid sequence of the pheromone (19) and yeast codon usage frequencies. The rationale is outlined in Fig. 1 where the last 5 amino acids of the α -factor and all the possible codons and their usage frequencies are given. (The codon usage is the total of 2 different glyceraldehyde-3-phosphate dehydrogenase clones (30, 31) and of alcohol dehydrogenase I.) The codon usage for these and other genes has recently been summarized (45). As can be seen from Fig. 1, virtually all possible sequences coding for the 5 amino acids are included in the oligonucleotide sequence 5'-GG^TCAACCA^AATGTAC. Accordingly, two pools consisting of two oligonucleotides each, and complementary to the above sequence, were synthesized. No other contiguous 5 amino acids in the pheromone could be covered with such a limited set of oligonucleotides.

F. Screening of Recombinant Plasmids

A genomic library, made by insertion of partially Sau3A-digested yeast DNA into the BamHI site of YRp7 (32), was screened for presence of α -factor gene clones. E. coli

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transformants were grown on nitrocellulose filter paper (Schleicher and Schuell, BA85) placed on S-agar plates containing 5 g/ml ampicillin. After 6 hours at 37°C, filters were transferred to S-agar plates containing 150 g/ml chloramphenicol. After 15 hours of amplification colonies were tested for hybridization using a modified in situ colony screening procedure (38). ³²P-labeled (40) synthetic oligonucleotides described above were used as hybridization probes. Filters were hybridized overnight at 42°C in 10mM Tris (pH 7.5), 6mM EDTA, 0.1mM ATP, 1mM sodium pyrophosphate, 0.8M NaCl, 1X Denhardt's solution, 0.5 percent NP-40, and 0.1 mg/ml E. coli tRNA. Filters were washed 3 times for 20 min. in 6XSSC at 30°. Dried filters were exposed to Kodak XR-2 X-ray film with Dupont Lightning-Plus intensifying screen at -80°.

G. Identification of Recombinant Plasmids Containing the α -factor Gene

Approximately 4500 bacterial colonies containing recombinant plasmids were tested for in situ hybridization (38) with ³²P-end-labeled oligonucleotide pool I (Fig. 1). Twenty-four plasmids hybridized to varying degrees. Small amounts of plasmid DNAs were prepared from these 24 colonies by the method of Birnboim and Doly (20) and tested for hybridization with the same probes after spotting the DNA samples on a nitrocellulose filter. Two of the 24 plasmids, designated as p51 and p52 respectively, hybridized strongly and were chosen for further study. The p51 and p52 plasmids also hybridized with the oligonucleotide pool II.

H. Subcloning of the Hybridizing Sequences

To characterize the inserts that hybridized with the synthetic probes, plasmid DNA prepared from the p51 and p52 clones was subjected to restriction enzyme analysis with EcoRI, Sall, HindIII, BamHI, and PstI. As seen in Fig. 2A, the 2 recombinant

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plasmids are quite dissimilar. Only EcoRI an PstI digestions of the two plasmids yielded one common fragment each. In both cases the common fragment is the TRP1 insert and the 1.38 PstI piece is the DNA between PstI sites in the TRP1 and the amp^R genes.

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The fragments that contained sequences complementary to the probe were identified by the method of Southern (42). Fig. 2B shows that, except in one case, digestion with all 5 restriction enzymes yielded a fragment that specifically hybridized with the probe. No hybridization was seen with any of the fragments produced by restriction of p52 DNA with HindIII.

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The smallest restriction fragments that contained sequences complementary to the synthetic probes were the 1.7 kbp EcoRI fragment from p52 and the 1.8 kbp HindIII fragment from p51. These two DNA fragments were isolated from a preparative agarose gel by electroelution and separately ligated to appropriately cleaved plasmid pBR322 (33) DNA. The ligation mixture was used to transform E. coli 294 and the plasmid DNA from the transformants was analyzed by a quick-screen procedure (20). Two transformants, designated p53 and p56, containing the 1.7 kbp EcoRI and 1.8 kbp HindIII fragment inserts, respectively, were analyzed as follows: Plasmid DNA was prepared from p53 an p56 and digested separately with BamHI, ClaI, PvuI, PstI, and SalI. The resulting DNA fragments were separated on a 1 percent agarose gel, transferred to nitrocellulose filter paper (42) and tested for hybridization with ³²p-labeled probes. The analysis of the restriction digests and corresponding hybridization patterns of the p53 DNA, the recombinant plasmid containing the 1.7 kbp yeast DNA as an EcoRI fragment, showed that the yeast DNA in this clone contained one SalI and two PstI sites and that the sequence complementary to the probes was included within a 0.5 kbp PstI-SalI fragment. The HindIII fragment of yeast DNA in the clone p56 lacked recognition sites for these enzymes, and the linearized plasmid, resulting from cleavage at single recognition sites for these enzymes in the pBR322 vector, hybridized with the probes.

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This plasmid was then digested with a number of additional restriction endonucleases and the digests were analyzed by the method of Southern as described above. It was found that the hybridizing sequences in this plasmid were contained on a 1.3 kbp HindIII-SacI fragment.

The property of growth inhibition of "a" cells by α -factor was used to test whether or not the pheromone gene contained in the cloned 1.7 kbp EcoRI and 1.8 kbp HindIII fragments are functional. If an active α -factor pheromone gene were present in a plasmid, it would be expected significantly more pheromone would be synthesized in cells containing the multi-copy plasmid than in cells containing only the chromosomal copy (or copies) of the gene. The enhanced level of the α -factor could then be detected by an increase in the area of nongrowth in a lawn of responsive "a" cells. The 1.7 kbp fragment, isolated from EcoRI-digested p53 DNA, and the 1.8 kbp fragment, isolated from HindIII-digested p56 DNA, were separately ligated to a pBR322-based vector plasmid which contained the yeast selectable marker TRP1 and the yeast origin of replication from the 2 μ m yeast plasmid (43). Yeast strain 20B-12 was separately transformed with these plasmids and with a control plasmid that lacked DNA sequences coding for the α -factor. The transformants were then compared for pheromone production. The transformants containing MF α 1 or MF α 2 coding sequences on plasmids produced significantly more α -factor than the same strain transformed with the control plasmid. We concluded that the 1.7 kbp EcoRI (MF α 1) and 1.8 kbp HindIII (MF α 2) fragments contain active α -factor pheromone genes. The result with MF α 1 is consistent with that described by Kurjan and Herskowitz (44), as this gene corresponds to the gene described by them.

I. DNA Sequence Determination

DNA sequence determination was as previously described

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(45). Briefly, DNA sequences were obtained by the chain termination method (47) using recombinant phages M13 mp8 and mp9 (39) as the source for single-stranded "template" DNA and a synthetic oligonucleotide for priming *E. coli* DNA polymerase I (large fragment, Boehringer Mannheim) in the presence of α -³²P dCTP (400 Ci/mmol, Amersham). Reactions were electrophoresed on 5 percent polyacrylamide/8M urea "thin" gels (47). Gels were dried onto 3MM paper (Whatman) and exposed to X-ray film for 2 to 12 hr.

The nucleotide sequences of large parts of the 1.7 kbp EcoRI fragment and the 1.3 kbp HindIII-SacI fragment are shown in Fig. 3 and Fig. 4, respectively. The p53 sequence contains an open reading frame coding for a protein of 165 amino acid residues which carries 4 internal repeat units within its C-terminal half. Each unit begins with Lys-Arg and ends with the α -factor sequence. Within each unit the pair of basic residues is separated from the α -factor by several Glu (or Asp)-Ala dipeptide repeats. The N-terminal half of the protein starts with a highly hydrophobic sequence of 22 amino acids which probably represents a signal sequence for secretion. The 61 amino acid residues between this hydrophobic sequence and the first repeat unit include 3 possible recognition sites for N-glycosylation (indicated by bars in figs. 3). The organization of the pheromone gene contained in p53 clone is identical to the MF α gene recently described by Kurjan and Herskowitz (44). This gene differs from MF α 1 at 4 positions. It contains T (instead of C) residues at positions -8 and -7, and 125 and an A (instead of C) residue at position 604. Because of the difference at position 125 there is a TTA (Leu) rather than TCA (Ser) codon at amino acid position 42. We have designated the gene contained in p53 as MF α 1.

A different α -factor gene, MF α 2, is present in the p56 clone. The organization of this gene (Fig 4) is similar, but not identical, to the MF α 1. The α -factor encoded by this gene is apparently made as a precursor protein of 120 amino acid residues

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containing two copies of the pheromone. One of the α -pheromone tridecapeptides contained in the putative precursor is identical to the pheromone copies encoded by the MF α 1 gene, whereas the second copy contains a Gln-->Asn and a Lys-->Arg.

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The organization of these precursors is strikingly similar to that of certain mammalian precursors for neuroendocrine peptides. Thus, like the proopiomelanocortin (48, 49), proenkephalin (50-52), and prodynorphin (53), the yeast precursors contain multiple peptide units destined for secretion. In all these precursors the secreted unit is contained on the C-terminal half of the precursor. The N-terminal half of the molecules carry possible glycosylation sites. As is the case for the mammalian multifunctional precursors, glycosylation may be involved in the correct processing of the α -factor precursor. However, the actual processing steps for the yeast precursor seem to be unpredictably different from those of mammalian precursor proteins. Whereas the pairs of basic residues (Lys-Arg) providing sites for release directly flank the secreted peptide in the mammalian precursors, cleavage at these sites in the α -factor precursor would release the pheromone units with several additional amino acids at the N-terminus (see figs. 3 and 4). These N-terminus extensions would consist of repeating -X-Ala- sequences in the precursors encoded by both MF α 1 and MF α 2 genes. Recent experiments (54, 55) indicate that the last step in the processing of the α -factor precursors is the removal of these sequences by dipeptidyl amino peptidases. The bee venom melittin (56) and the frog skin caerulein (57) precursors are apparently processed by similar mechanisms.

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J. Construction of a Plasmid for Expression and Secretion of Human Interferon

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Although, as discussed above, our DNA sequence data suggest that the α -factor is synthesized as precursor proteins of 165 and 120 amino acids, no such proteins have been described. The

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processing and secretion mechanism of α -factor is not known. Recent studies, however, with altered α -factor indicate that the last step in the production of mature α -factor is apparently the removal of the glu-ala or asp-ala units before the release of the α -factor oligopeptides of 12-13 amino acids having the basic sequence
5 $H_2N-(Trp)-His-Trp-Leu-Gln-Leu-Pro-Gly-Gln-Pro-Met$ (or
 $MetSO)-Tyr-COOH$.

The preparation of a plasmid to demonstrate the usefulness
10 of the α -factor promoter and the α -factor presequences for
expression and secretion of heterologous gene products is outlined
in Fig. 5. The DNA sequences coding for the α -factor presequences
for expression and secretion of heterologous gene products is
outlined in Fig. 5. The DNA sequences coding for the α -factor
15 peptides were removed from one of the α -factor clones (p53) such
that the resulting plasmid, p57, contained the promoter sequences
and the sequence corresponding to 89 amino acids of the α -factor
"prepro" protein. This sequence was then joined with human
interferon D ($IFN-\alpha_1$) gene to form plasmid p58. The human
20 interferon D gene (58) was modified such that DNA sequences
corresponding to Leu-Glu-Phe had been added before the initiating
methionine codon. After modified interferon D gene had been joined
with the α -factor "prepro" and the promoter sequences, these
sequences were isolated and inserted into a yeast-E. coli shuttle
25 plasmid YEp9T (Fig. 6). The plasmid YEp9T had been previously made
by replacing the EcoRI-SalI fragment in plasmid YEp1PT (59) with the
EcoRI-SalI fragment from pBR322. This plasmid contains the pBR322
(33) DNA needed for its selection and replication in E. coli. In
addition, it contains the yeast TRP1 gene on an EcoRI to PstI
30 fragment from chromosome IV (34-36) and a yeast origin of
replication on a PstI to EcoRI fragment from the endogenous 2μ
plasmid DNA. These two DNA fragments from yeast allow for its
selection in yeast and for its autonomous replication and
maintenance as a plasmid. The resulting plasmid, p60, with the
35 indicated orientation of the insert was selected because the 2μ

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origin contains a transcription termination/polyadenylation signal (37). The DNA sequence at the junction of the α -factor "prepro" sequence and the modified LeIFN-D gene present in p60 is shown in Fig. 7. The p60 plasmid was introduced into the yeast strain 20B-12 and the trp^+ transformants were grown and assayed for interferon production.

K. Interferon Assay of Growth Medium and Cell Extracts

Individual colonies of the transformants were grown at 30°C in 20 ml YNB+CAA to an A_{660} of approximately 10. For assay 10 ml aliquot was centrifuged at 7K rpm for 10 minutes in a Sorval SM24 rotor. Various dilutions of supernate (media) were assayed. The cells were resuspended in 0.5 ml 7M guanidine-HCl containing an equal volume of glass beads and vortexed for 2 minutes at high speed. Both the cell lysate and the medium were then diluted into PBS/BSA (150 mM NaCl, 20 mM sodium phosphate (pH = 7.9), and 0.5 percent bovine serum albumin) for bioassay. Extracts of yeast were assayed for interferon by comparison with interferon standards by the cytopathic effect (CPE) inhibition assay (26). Up to one hundred million units of interferon per liter of growth medium was found. The cell extracts also yielded interferon at the rate of 100×10^6 units per liter of culture.

L. Purification of Interferon from the Medium

A single colony of yeast strain p60/20B-12 was grown at 30°C in 500 ml YNB+CAA to an A_{660} of 2.4. Five hundred ml of this culture was diluted to 5L with YNB+CAA to give an A_{660} of 0.21; the resultant 5L culture was grown at 30°C until $A_{660} = 70$. At this time the 5L culture was harvested by centrifugation at 7,000 rpm for 10 minutes. Ten ml aliquots were withdrawn periodically during the fermentation to measure optical density, interferon production and secretion. Before assay, each aliquot was

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centrifuged for 5 minutes in a bench-top refrigerated centrifuge to separate the cells from the medium. The medium and cells were assayed as described above (see Fig. 8). Two different fermentations were done. The peak activity of interferon in the media were 3×10^9 and 2×10^9 units per liter, respectively. The interferon activity in the cell extracts were 1×10^9 and 2×10^9 units per liter of culture.

One and a half liters of frozen medium were concentrated and dialyzed against 25mM Tris, 10mM EDTA, pH 8.0 in a 2.5 liter Amicon stirred cell (Amicon 2000) using a YM-5 ultrafiltration membrane to a final volume of 116 ml. A sample of the retentate was sequenced directly. Another sample of the retentate was acetone precipitated and sequenced.

One ml of the concentrated medium was precipitated with 4 ml acetone, spun in a microfuge and washed with acetone. The pellet was resuspended in 0.1 percent TFA and further purified by HPLC on a Synchropak RP-P column. The column was eluted with a linear gradient of 0 to 100 percent acetonitrile in 0.1 percent TFA in 60 minutes. A 12 μ g sample of purified IFN- α AD was chromatographed as a control. The peaks of absorbance at 280 nm were sequenced.

M. N-terminal Amino Acid Sequence of Interferon-from Growth Medium

Sequence analysis was based on the Edman degradation (27). Liquid samples were introduced into the cap of a modified Beckman 890B spinning cap sequencer. PolybreneTM was used as a carrier in the cap (28). Reagents used were Beckman's sequence grade 0.1 molar Quadrol buffer, phenyl-isothiocyanate, and heptafluorabutyric acid. Norleucine was added during each cycle with the Quadrol buffer to serve as an internal standard. The presence of PTH-norleucine in each chromatogram aided in the identification of PTH amino acids by retention time. The amino acid sequence analysis showed only one

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species of interferon molecule with the NH₂-terminal sequence NH₂-Glu-Ala-Glu-Ala-Leu-Glu-Phe-Met. The Met results from the start codon at the N terminus of the interferon gene, and thus the protein produced contains 7 extra amino acids, three from the construction, i.e., Leu-Glu-Phe (see figure 7) and 4 from the presequence of α -factor, i.e., Glu-Ala-Glu-Ala. The polypeptide containing this 7 amino acid N-terminal extension retains interferon activity.

N. Expression and Secretion of Other Heterologous Gene Products

In the process of testing the utility of the α -factor promoter and "prepro"-sequence, restriction endonuclease sites were created at the end of the α -factor "prepro"-sequence (see Fig. 7) such that the promoter and the "prepro"-sequence could be isolated as a portable restriction fragment. An appropriate plasmid could then be constructed to test the efficacy of this expression and secretion system for any heterologous gene containing suitable "sticky" ends. For this purpose an expression plasmid p65, was constructed as shown in Fig. 9. This plasmid, like YEp9T, contains the origins of replication for E. coli and yeast as well as selective markers for selection in each; of these two organisms. It also contains a convenient EcoRI site for gene insertion so that any gene that is contained on an EcoRI fragment where the first codon of the gene is immediately preceded by the EcoRI site could be tested for the synthesis and secretion of the corresponding protein.

The plasmid p65 was partially digested with EcoRI the linear molecules isolated, and ligated with EcoRI fragments containing various genes. After transformation of E. coli, plasmids that contained the inserts in the appropriate orientation were selected. For expression the fragment must be inserted at the EcoRI site following the promoter with the 5' end of the gene connected to that site. This orientation creates the junction between the α -factor

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signal sequence and the heterologous gene as previously shown for LeIF-D (see Fig. 7).

Table I lists genes that have been thus tested with the α -factor promoter and signal sequence.

TABLE I Expression and Secretion of Other Heterologous Genes Using the α -Factor Promoter and Signal Sequence

Gene	Growth Vessel	Products per Liter Cellular	Medium
Human Interferon γ	Shake-flask	10^5 units	trace
Human Serum Albumin	Fermentor	25 mg	3 mg
Bovine Interferon $\alpha 1$	Fermentor	100×10^6 U	200×10^6 U
Bovine Interferon $\alpha 2$	Fermentor	400×10^6 U	60×10^6 U
Tissue Plasminogen Activator	Shake-Flask	20 μ g	20 μ g
Rennin	Shake-Flask	100 μ g	trace
Human Insulin-Like Growth Factor	Fermentor	1-5 mg	3 mg

The expression of the first 4 genes was achieved by the insertion of EcoRI fragments into p65 as described above. The genes were obtained by EcoRI digestion of plasmids containing them as described in US 438,128 (GB 2116566, EP 88622) (BoIFN); US 297,380 (GB 2105343, EP 73646) (HSA); and US 312,489 (GB 2107718, EP 77670) (γ IFN) and elsewhere, e.g. in Interferons edited by Merigan, et al., Academic Press, Inc. (1982), Proceedings of the Symposium on "Chemistry and Biology of Interferons: Relationship to Therapeutics", held March 8-12, 1982, Squaw Valley, California; Lawn, et al. Nucleic Acids Research 9, 6103 (1981); Gray, et al., Nature 295, 503 (1982).

Because of the placement of the restriction sites in the t-PA and rennin genes it was not practical to construct expression plasmids directly as above, but a modified approach was taken. The

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construction of the t-PA expression plasmid is illustrated in Fig. 10 using plasmid pt-PATrpl2 (60) to obtain the t-PA gene by excision with XbaI and BglII, pBI (described in US 438,236 (EP 88632), supra) and YEp13 (43). The rennin expression plasmid was assembled in analogous manner using the rennin gene obtained by XbaI-BclI excision of pRI (described in US 452,227, EP application 83307841.3). These two plasmids contain the LEU2 gene for selection in yeast. Therefore an α leu2 yeast strain was used for transformation with these plasmids. The prorennin expression plasmid pRI was 10 constructed by incubating EcoRI-Pst I cleaved γ -Imm plasmid with 5' and 3' segments in the presence of T4 ligase. γ -IMM is a pBR 322 derived plasmid described in U.S. Appln. Serial No. 312,489, (GB 2107718, EP 77670) and Gray, et al., Supra.

15 The 5' end fragment was a ligation of a synthetic fragment and a fragment derived from clone PFLA, as described below. The synthetic fragment has the structure:

20 [Met] [Ala] [Glu] [Ile] [Thr]
d A A T T C A T G G C A G A A A T A A C A A G
 G T A C C G T C T T T A T T G T T C C T A G d
reading direction--->

25 This sequence contains an EcoRI site at the "upstream end" an ATG start codon, followed by sequences coding for the first four amino acids in prorennin, and terminates in a BamHI site.

30 The cloned fragment to which this synthetic fragment is ligated in order to form the 5' end of the gene comprises a Xma-BamHI fragment of approximately 440 base pairs derived from the 5' end of the gene.

35 The appropriate 440 bp fragment was obtained from cDNA derived from unfractionated mRNA, using as primer, α GATCCGTCGAATTCGG, i.e., the "primer probe". The cDNA formed using this primer was size fractionated as set forth above and fragments having more than 1,000

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base pairs inserted into the PstI site of pBR322 for cloning. The resulting clones were selected using both Tth probe and the primer/probe as probes. Only colonies hybridizing with both were selected. From 1,280 transformed colonies, about 300 colonies were obtained which showed hybridization with both probes. These were examined for presence of the 5' portion of the prorennin sequence as follows:

The results of a series of double digestions using Ava I-Pvu I, 10AvaI-BamHI, BglIBamHI, and BglI-EcoRI were analyzed. Advantage was thus taken of the known Pvu I and Bgl I sites, each 125 base pairs either side of the pBR322 Pst site utilized for insertion of the cDNA sequence. These digestions provide suitable fragments for analysis.

15

The desired clone, PFLA, was selected by analysis of acrylamide gel electrophoresis performed on the above double digests of mini preps prepared from the identified clones. Plasmids were then isolated from PFLA clone, double digested with BamH I and XmaI, and 20the 440 bp fragment recovered by gel electrophoresis.

The "complete" 5' end was then created by a standard ligation reaction utilizing the synthetic fragment and the PFLA clone BamHI-XmaI fragment with T4 ligase followed by cleavage with XmaI 25and EcoRI. The resulting ligated sequences were purified on acrylamide gel electrophoresis selecting for the appropriate 455 base pair fragment.

The 3' end fragment was prepared in an manner analogous to that 30used to prepare the PFLA clone. cDNA containing >1000 bp formed from unfractionated messenger RNA using oligo-dT as primer, was cloned as above, and colonies selected with Tth probe. Approximately 50 colonies resulted. The desired clone was selected by analyzing the results of gel electrophoresis formed on plasmid minipreps which 35were double digested with BamHI/BglI, PvuI/BamHI, EcoRI/BglI, and

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PvuI/EcoR I again taking advantage of the PvuI and BglI sites flanking the PstI insert site. The plasmids from the desired colony were isolated then cleaved with Xma I and Pst I and electrophoresed to isolate the 800 bp sequence of the "3 - 375" fragment. The "3-375" fragment extends from the Xma site congruent with that from the PFLA fragment, past the end of the gene to the Pst I insertion site.

The transformants carrying various expression plasmids were grown in appropriate media. The cultures were fractionated into supernatants and cells. The supernatants (media) and cell extracts were assayed for the expression and secretion of various gene products. The bovine interferon activity was assayed by comparison with interferon standards by cytopathic effects. The amounts of other products in the medium and cell extracts were determined by radioimmunoassays. The values listed in the table are the peak activities. We have determined that both cellular and secreted t-PA molecules possess biological activity.

20

0. Characterization of Secreted Bovine Interferon

The bovine interferon- α_1 secreted into culture medium has been purified and characterized as described below.

25

7 liters of culture medium was adjusted to pH 8.02 with sodium hydroxide. This solution was then loaded onto a 2.5 x 18 cm Nuge! ACA column pre-equilibrated with 50mM tris, pH 8.0. After loading, the column was washed with 50mM Tris, 1 percent (w/v) PEG 8000, pH 8.0 until A_{280} was approximately zero. The column was then eluted with 100mM ammonium acetate, 2 percent (w/v) PEG 8000, pH 5.0 followed by 20mM glycine, 2.5 percent (w/v) PEG 8000, pH 2.0. The majority of the interferon activity eluted in a single peak during the pH 5.0 elution. The pooled material from the Nuge! column (83 35mL, 150M units) was loaded onto a 2.5 x 5.0 cm SE-53 column

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pre-equilibrated with 25mM ammonium acetate, pH 5.0. The interferon activity eluted in a single peak during the sodium chloride elution. This pool contained 14mg protein, 106M units in 11 mL. The purity of this material as judged by SDS PAGE was approximately 80-90 percent.

Ten ml of the SE-53 pool was applied to a 2.5 x 18 cm Sephacryl S-300 column equilibrated in 25mM sodium phosphate, pH 6.0. The column was eluted with this buffer and the interferon activity eluted in a single peak. This final pool contains 8 mg protein, 113 M units in 17 mL.

N-terminal sequence analysis of the protein present in the SE-53 pool indicates that the bovine interferon secreted by yeast has been processed at three distinct sites. The three sites of processing and the relative amounts of each are as follows:

-8	-6	-3	+1
...lys-arg-glu-ala-glu-ala-leu-glu-phe-met-cys-his-leu-pro-his...			
63 pct.	13 pct.	24 pct.	

As shown above, met immediately precedes the N-terminus of the bovine interferon; the short peptide extensions do not result in loss of interferon activity.

Production and Secretion of Mature Heterologous Proteins

In both cases (human IFN- α_1 , and bovine IFN- α_1) where the N-terminal amino acid sequence of the secreted polypeptides was determined, the proteins contained extensions of 2 to 7 amino acids in addition to the initiating methionine. Although these polypeptides have biological activity, it would be preferable to produce and secrete into the growth medium proteins that are identical to the proteins from the natural sources.

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In order to produce the interferon molecule that contains cysteine as the N-terminal amino acid (as is the case with the natural IFN- α_1) we needed to modify the junction between the factor "pre-pro" sequence and the IFN- α_1 gene such that the removal of the modified "pre-pro" sequence will result in release of a mature interferon molecule containing the natural N-terminus. An outline of the procedure to obtain such a junction is shown in Figure. 11. A DNA fragment containing the HpaI promoter and "pre-pro" sequence and the modified IFN- α_1 gene was isolated and cloned into the EcoRI site of M13 mp8 (61). Single-stranded DNA template was prepared from the recombinant phage containing the insert in the appropriate orientation. This template was annealed with a phosphorylated oligonucleotide. The synthetic oligonucleotide is 24 bases long and is complementary to 12 bases coding for leu-asp-lys-arg near the C-terminus of the "pre-pro" sequence and to 12 bases coding for cys-asp-leu-pro, the first 4 amino acids of natural IFN- α_1 . This primer-templated intermediate was subjected to extension and ligation reaction at 23°C for 2 hours in the presence of 500 mM dATP, 100 mM dTTP, 100 mM dGTP, 100 mM dCTP, 20 mM dATP, 3 units DNA polymerase (Klenow), and 400 units T4 DNA ligase in 10 mM Tris pH7.4, 50 mM NaCl and 10 mM Mg 504. Then additional 3 units of DNA polymerase (Klenow) and 400 units of T4 DNA ligase was added and mixture incubated for 2 hours at 23°C followed by incubation at 14°C for 15 hours. This mixture was used to transform E. coli JM101 (62). The phage plaques were screened for hybridization with the ³²P-labeled oligonucleotide. Templated DNA from 2 positive recombinant phages was prepared and sequenced using a primer complementary to IFN- α_1 DNA. Double stranded DNA from one recombinant phage that contained the desired deletion (deletion of 24 nucleotides shown as a loop in Fig. 11) was prepared. The EcoRI fragment containing the modified junction between the "pro-pro" sequence and the IFN- α_1 gene was isolated and ligated to EcoRI cleaved YEP9T. After transformation of E. coli with the mixture a plasmid (p76) with correct orientation of the insert was chosen for further study.

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p76 DNA was prepared from E. coli and used to transform 208-12 strain (ATCC 20626) of yeast. Ten liter culture of one transformant was grown and the culture medium was centrifuged to separate the medium from the cells.

5 500 ml of yeast medium was dialyzed into 25 mM Tris, pH8.0, 10 mM EDTA. The dialyzed media was then run through an immuno affinity column containing monocloned antibody to nature (LeIFA). After washing with 24 mM TRIS pH8.0, 10 mM EDTA, the intrferon activity
10 was eluted with 0.2M acetic acid. The majority of interferon activity was found in Fraction No. 45. 200 μ l of this fraction was subjected to N-terminal amino acid sequence analysis as described before. The major sequence found was that of natural interferon D. The first 8 N-terminal amino acids of the protein were:
15 cys-asp-leu-pro-glu-thr-his-ser.

Additional Explanatory Notes re Figures

Figure 2

20 Localization of homology between the α -factor probes and the DNA fragments from p51 (a) and p52 (b) recombinant plasmids. The two plasmids were digested with different restriction endonucleases and then electrophoresed on a one percent agarose gel. The DNA
25 fragments were transferred to nitrocellulose paper and hybridized to ³²P-labeled probes. Panel A: Ethidium bromide stained gel.
Panel B: Southern blot. Lanes: 1, EcoRI; 2, SallI; 3, HindIII; 4, BamHI; 5, PstI. The arrows indicate the two DNA fragments that were subcloned. The size standards were derived from lambda, YRp7, or
30 pBR322 DNA.

Figures 3

35 Nucleotide sequence of MF α_1 gene and its nontranslated 5' and

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3' flanking regions. The predicted amino acids sequence of the pheromone precursor is also shown. The numbers above and below the sequence denote the positions of amino acids and nucleotides, respectively. The four copies of the α -factor sequences are included in the boxed areas. The asterisks indicate differences in the nucleotide sequence in one or more copies of the α -factor coding regions. Three potential N-glycosylation recognition sites are indicated by bars. This gene corresponds to the gene reported by Kurjan, et al. (44).

Figure 4

Nucleotide sequence of MF α 2 gene and its nontranslated 5' and 3' flanking regions. The underlined amino acids indicate differences between the two pheromone copies encoded by the MF α 2 gene. See Fig. 3 for other details.

Figure 5

Joining of the IFN- α_1 gene with the α -factor promoter and the α -factor presequence. Since, as shown in Fig. 3, the 1.8 kbp EcoRI fragment contained the promoter, the entire DNA sequence 5' to the sequences coding for the mature α -factor was joined to the modified IFN- α_1 gene such that the α -factor presequence and the IFN- α_1 protein would be synthesized as a single precursor protein using the α -factor promoter.

Figure 7

The protein and DNA sequence at the junction of α -factor "prepro" and the modified IFN- α_1 genes. The XbaI and EcoRI sites at the junction are indicated.

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Figure 8

Cellular and medium IFN- α_1 levels during fermentation. At various time intervals 10 ml culture was removed from the fermentor, centrifuged to separate the cells and medium. Cell extracts were prepared as described above. Interferon levels in the medium and the extracts were determined.

Figure 9

Construction of a yeast/E. coli shuttle vector plasmid for the expression of heterologous genes using the α -factor promoter and signal sequences. The 1.12 kbp EcoRI fragment containing the promoter and signal sequence was isolated from p58 and was inserted into the EcoRI site of YEp9T.

Figure 10

Assembly of plasmid p68 for expression and secretion of tissue plasminogen activator.

Figure 11

Scheme for in vitro deletion mutagenesis. The 24 nucleotides that were deleted at the junction of MF21 "pre-pro" sequence and the modified IFN- α_1 gene are shown as a loop in the figure.

Figure 12

Comparison of amino acid sequences of the putative α -factor precursors encoded by MF α_1 and MF α_2 genes. Various gaps were created to align the sequences with maximum homology.

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Bibliography

1. Novick, et al., Cell 21, 205 (1980).
2. Duntze, et al., Science 168, 1472 (1970).
- 5 3. Woods, et al., J. Gen. Microbiol. 51, 115 (1968).
4. Cabib, et al., J. Bacteriology 124, 1586 (1975).
5. Farkas, Microbiol. Rev 43, 117 (1979).
- 10 6. Moor, Arch. Mikrobiol. 57, 135 (1967).
7. Goeddel, et al., Nature 287, 411 (1980).
8. Goeddel, et al., Nature 290, 20 (1981).
9. Yelverton, et al., Nucleic Acids Research 9, 731 (1981).
- 15 10. Goeddel, et al. Nucleic Acids Research 8, 4057 (1980).
11. Wetzel, American Scientist 68, 664 (1980).
12. Wetzel, et al., Biochemistry 19, 6096 (1980).
13. Davis, et al., Proc. Natl. Acad. Sci. (USA) 78, 5376 (1981).
- 20 14. Hitzeman, et al., Nature 293, 717 (1981).
15. Kleid, et al., Science 214, 1125 (1981).
16. Lawn, et al., Nucleic Acids Res. 9, 6103 (1981).
17. Weck, et al., Nucleic Acids Res. 9, 6153 (1981).
- 25 18. Stotlzer, et al., Eur. J. Biochem. 65, 257-262 (1976).
19. Stotlzer, et al., Eur. J. Biochem. 69, 397-400 (1976).
20. Birnboim, H.C., et al., Nucleic Acids Research 7, 1513 (1979).
21. Hinnen, A., et al., Proc. Natl. Acad. Sci. (USA) 75, 1929 (1978).
- 30 22. Backman, et al., Proc. Natl. Acad. Sci (USA) 73, 4174- (1976).
23. Mandel, et al., J. Mol. Biol. 53, 159 (1970).

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-31-

24. Beggs, Nature 275, 104 (1978).
25. Miller, J.H., Experiments in Molecular Genetics, pp. 431, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. Stewart, W.E. II The Interferon System (Springer, New York, 1979).
27. P. Edman, G. Begg, "A Protein Sequencer" Eur. J. Biochem. 1, 80 (1967).
28. Tarr, G.E., et al., Anal. Biochem. 84, 622 (1978).
29. Clernell, et al., Biochemistry 9, 4428 (1970).
30. Holland, et al., J. Biol. Chem. 254, 9839 (1979).
31. Holland, et al., J. Biol. Chem. 255, 2596 (1980).
32. Struhl, et al., Proc. Natl. Acad. Sci. USA 76, 1035 (1979).
33. Bolivar, F., et al., Gene 2, 950113 (1977).
34. Stinchcomb, D.T., et al., Nature 282, 39 (1979).
35. Kingsman, et al., J. Gene 7, 141 (1979).
36. Tschumper, G., et al., Gene 10, (1980).
37. Hartley, J.L. et al., J.E. Nature 286, 860 (1980).
38. Grunstein, et al., Proc. Natl. Acad. Sci. USA 72, 3961 (1975).
39. Messing, et al., Nucleic Acids Research 9, 309 (1981).
40. Maxam, A.M., and Gilbert, W., Methods in Enzymol. 65, 490 (1980).
41. Crea, R. and Horn, T. Nucleic Acids Res. 8, 2331 (1980).
42. Southern, J. Mol. Biol. 98, 503 (1975).
43. Broach, et al., Gene 10, 157 (1979).
44. Kurjan, et al., Cell 30, 933 (1982).
45. Smith, Methods Enzymol. 65, 499 (1980).
46. Bennetzen, J.L., et al., J. Biol. Chem. 257, 3026 (1982).
47. Sanger, et al., Proc. Natl. Acad. Sci. USA 74, 5463 (1977).

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-32-

48. Mains, et al., Proc. Natl. Acad. Sci. USA 74, 3014 (1977).
49. Hakanishi, et al., Nature 278, 423 (1979).
50. Comb, et al., Nature 295, 663 (1982).
- 5 51. Gubler, et al., Nature 295, 206 (1982).
52. Noda, et al., Nature 295, 202 (1982).
53. Kokidani, et al., Nature 298, 245 (1982).
- 10 54. Barnes, et al., Berkeley Workshop on Recent Advances In Yeast Molecular Biology (1982): Recombinant DNA, Esposito, et al. Eds., pp. 295-305, Lawrence Radiation Laboratory, University of California, Berkeley, CA.
55. Julius, et al., Cell 32, 839 (1982).
56. Kreil, et al., Eur. J. Biochem. 111, 49 (1980).
- 15 57. Hoffman, et al., EMBO J. 2, 111 (1983).
58. Weck, et al., Nuc. Acid Res. 9, 5153 (1981).
59. Hitzeman et al., Science 219, 620 (1983).
60. Pennica, et al., Nature 301, 214 (1983).
- 20 61. Messig, J. (1981) pp. 143-153 in Third Cleveland Symposium on Macromolecules: Recombinant DNA, Ed. A. Walton, Elsevier, Amsterdam.
62. Messig, J., Recombinant DNA Technical Bulletin 2, 43 (1979).

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Claims:

1. A process for obtaining a protein heterologous to yeast as a product of yeast expression, which comprises:

transforming a yeast organism with a yeast expression vehicle comprising the DNA sequence of the promoter for yeast alpha factor operably connected to a DNA sequence encoding a protein heterologous to the yeast organism;

culturing the transformed organism; and
recovering the protein from the culture.

2. A process for obtaining a protein heterologous to yeast as a product of yeast expression, which process comprises:

transforming a yeast organism with an expression vehicle comprising the DNA sequence encoding substantially the pre-pro peptide of yeast alpha factor operably connected in translation reading frame to a DNA sequence encoding a mature protein heterologous to the yeast organism;

culturing the transformed organism; and
recovering the protein from the culture.

3. A process for obtaining a protein heterologous to yeast as a product of yeast expression, processing and secretion, which process comprises:

transforming a yeast organism with an expression vehicle comprising the DNA sequence of the promoter operably linked to substantially the pre-pro peptide sequence of the yeast alpha factor.

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gene which is operably connected in translation reading frame to a DNA sequence encoding a mature protein heterologous to the yeast organism;

5 culturing the transformed yeast organism; and
 recovering protein from its supporting medium.

4. A process for secreting a protein heterologous to yeast into the supporting medium, which process comprises:

10 transforming a yeast organism with an expression vehicle
 comprising the DNA sequence encoding substantially the pre-pro
 peptide of yeast alpha factor, operably connected in translation
 reading frame to a DNA sequence encoding a mature protein
15 heterologous to the yeast organism; and

 culturing the transformed organism.

20 5. A process of Claim 4 wherein said DNA sequences are
 under the control of alpha factor promoter.

25 6. A yeast expression vehicle comprising the DNA sequence
 of the promoter of the yeast alpha factor gene operably connected to
 a DNA sequence encoding a protein heterologous to the yeast organism.

30 7. An expression vehicle of Claim 6 which also includes
 the DNA sequence encoding substantially the pre-pro peptide of yeast
 alpha factor operably linked in translation reading frame upstream
 to the DNA sequence encoding a mature protein heterologous to the
 yeast organism.

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8. A yeast expression vehicle comprising the DNA sequence encoding substantially the pre-pro peptide of yeast alpha factor gene operably connected in translation reading frame to a DNA sequence encoding a mature protein heterologous to the yeast organism.

9. An expression vehicle of Claims 6 to 8 wherein the DNA encoding heterologous protein s.a.g. encodes for a protein selected from the group consisting of human interferon, bovine interferon, tissue plasminogen activator, and rennin.

10. An expression vehicle of Claims 6 to 8 wherein the DNA encoding heterologous protein encodes for insulin-like growth factor.

11. A yeast organism transformed with an expression vehicle of any one of Claims 6 to 10.

12. A yeast organism capable of producing mature heterologous protein in the supportive medium, as a product of expression with a N-terminus pre-sequence derived from yeast alpha factor DNA, processing of said pre-sequence and secretion of the mature protein into the medium.

13. The organism of Claim 12 wherein the mature heterologous protein is human insulin-like growth factor.

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Fig.1.

Carboxy terminus
of α -factor:

Gly -- Gln -- Pro -- Met -- Tyr COOH

Possible codons
and their usage

GGU (90)	CAA (20)	CCA (32)	AUG (20)	UAC (33)
GGC (3)	CAG (0)	CCU (3)		UAU (0)
GGA (0)		CCC (1)		
GGG (0)		CCG (0)		

Consensus
oligonucleotides:

5'-GG^T_CCAACC^A_TATGTAC

Synthesized
oligonucleotide
pools complemen-
tary to above:

I. 5'-GTACATTGGTTG^A_GCC
II. 5'-GTACATAGGTTG^A_GCC

Fig.7.

ALPHA-FACTOR "PRE-PRO" SEQUENCE

MODIFIED IFN-ALPHA₁ GENE

...LYS ARG GLU ALA GLU ALA

LEU GLU PHE MET...

...AAA AGA GAG GCT GAA GCT

CTA GAA TTC ATG...

...TTT TCT CTC CGA CTT CGA

GAT CTT AAG TAC...

XbaI

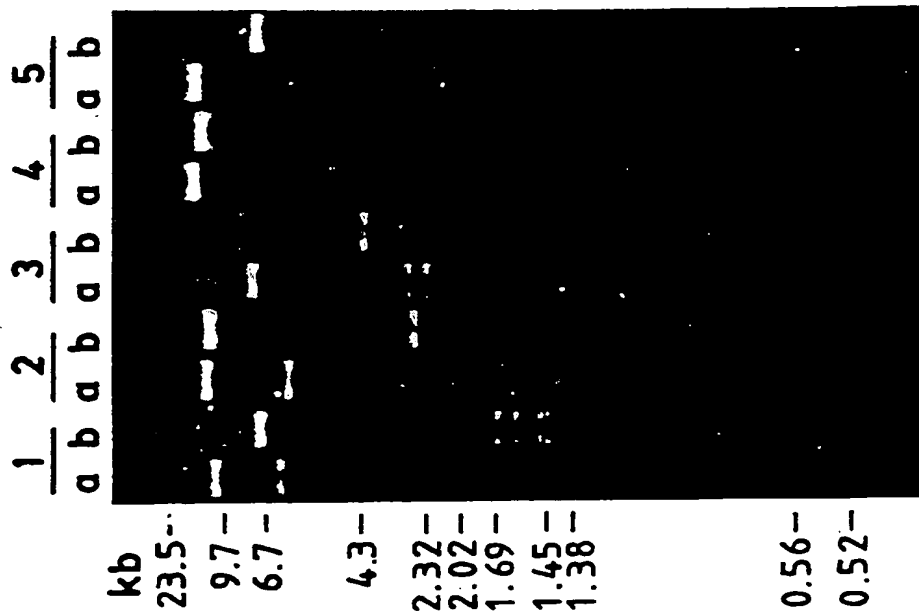
EcoRI

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Fig.2.

A



B



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3//
Fig.3.

CGACAGTAAATTTGCCGAATTTAATAGCTTCTACTGAAAAACAGTGGACCATGTGAAAAGATGCATCTCATTTATCAA
-280 -260 -240 -220

ACACATAATATTCAAGTGAGCCTTACTTCAATTGTATTGAAGTGAAGAAAACCAAAAAGCAACAACAGGTTTTGGATA
-200 -180 -160 -140

AGTACATATATAAGAGGGCCTTTTGTCCCATCAAAAATGTTACTGTTCTTACGATTCAATTTACGATTCAAGAATAGTT
-120 -100 -80 -60

CAAACAAGAAGATTACAACTATCAATTTATACACAAATATAAACGATTAAAAGA ATG AGA TTT CCT TCA ATT
-40 -20 1
Met Arg Phe Pro Ser Ile
1

Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr
TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA
20 40 60

Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Leu Asp Leu Glu Gly
GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TTA GAT TTA GAA GGG
80 100 120

Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe Ile
GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT ATA
140 160 180

Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu
AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT TTG GAT AAA AGA GAG
200 220 240

Ala Glu Ala Trp His Trp Leu Gln Leu Lys Pro Gly Gln Pro Met Tyr Lys Arg Glu Ala
GCT GAA GCT TGG CAT TGG TTG CAA CTA AAA CCT GGC CAA CCA ATG TAC AAG AGA GAA GCC
260 280 300

Glu Ala Glu Ala Trp His Trp Leu Gln Leu Lys Pro Gly Gln Pro Met Tyr Lys Arg Glu
GAA GCT GAA GCT TGG CAT TGG CTG CAA CTA AAG CCT GGC CAA CCA ATG TAC AAA AGA GAA
320 340 360

Ala Asp Ala Glu Ala Trp His Trp Leu Gln Leu Lys Pro Gly Gln Pro Met Tyr Lys Arg
GCC GAC GCT GAA GCT TGG CAT TGG CTG CAA CTA AAG CCT GGC CAA CCA ATG TAC AAA AGA
380 400 420

Glu Ala Asp Ala Glu Ala Trp His Trp Leu Gln Leu Lys Pro Gly Gln Pro Met Tyr End
GAA GCC GAC GCT GAA GCT TGG CAT TGG TTG CAG TTA AAA CCC GGC CAA CCA ATG TAC TAA
440 460 480 165

GCCCCGACTGATAACAACAGTGTAGATGTAACAAAGTCGACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAAT
500 520 540 560

ATACTTTTCATTTCTCCGTAACAACATGTTTTCCCATGTAATATCCTTTTCTATTTTGGTTCCGTTACCAACTTTAC
580 600 620 640

ACATACTTTATAGCTATTCACTTCTATACACTAAAAAACTAAGACAATTTTAATTTTGCTGCCTGCCATATTTCAAT
660 680 700 720

TTGTTATAAATTCCTATAATTTATCCTATTAGTAGCTAAAAAAGATGAATGTGAATCGAATCCTAAGAGAATTC
740 760 780 800

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Fig.4.

TTCTTCATTGGTACATCAATGCCAGCAACGATGTGCGCATCTGGGCGACGCCTGTAGTGATTGTTTTCAAGGTATCGAG
-300 -280 -260 -240

CCAACTATTTCATCGTTACTGTTTCAAATATTCAGTTGTTTCAGTACAGAGTCGCCGTGGACCTAGTGAACTTGGTGT
-220 -200 -180 -160

CTTTACAGCGCAGAGACGAGGGCTTATATGTATAAAAGCTGTCCTTGATTCTGGTGTAGTTTGAGGTGTCCTTCCTATA
-140 -120 -100 -80

TCTGTTTTTATATTCTATATAATGGATAATTACTACCATCACCTGCATCAAATTCAGTAAATTCACATATTGGAGAAA
-60 -40 -20

1 10 20
Met Lys Phe Ile Ser Thr Phe Leu Thr Phe Ile Leu Ala Ala Val Ser Val Thr Ala Ser
ATG AAA TTC ATT TCT ACC TTT CTC ACT TTT ATT TTA GCG GCC GTT TCT GTC ACT GCT AGT
1 20 40 60

30 40
Ser Asp Glu Asp Ile Ala Gln Val Pro Ala Glu Ala Ile Ile Gly Tyr Leu Asp Phe Gly
TCC GAT GAA GAT ATC GCT CAG GTG CCA GCC GAG GCC ATT ATT GGA TAC TTG GAT TTC GGA
80 100 120

50 60
Gly Asp His Asp Ile Ala Phe Leu Pro Phe Ser Asn Ala Thr Ala Ser Gly Leu Leu Phe
GGT GAT CAT GAC ATA GCT TTT TTA CCA TTC AGT AAC GCT ACC GCC AGT GGG CTA TTG TTT
140 160 180

70 80
Ile Asn Thr Thr Ile Ala Glu Ala Ala Glu Lys Glu Gln Asn Thr Thr Leu Ala Lys Arg
ATC AAC ACC ACT ATT GCT GAG GCG GCT GAA AAA GAG CAA AAC ACC ACT TTG GCG AAA AGA
200 220 240

90 100
Glu Ala Val Ala Asp Ala Trp His Trp Leu Asn Leu Arg Pro Gly Gln Pro Met Tyr Lys
GAG GCT GTT GCC GAC GCT TGG CAC TGG TTA AAT TTG AGA CCA GGC CAA CCA ATG TAC AAG
260 280 300

110 120
Arg Glu Ala Asn Ala Asp Ala Trp His Trp Leu Gln Leu Lys Pro Gly Gln Pro Met Tyr
AGA GAG GCC AAC GCT GAT GCT TGG CAC TGG TTG CAA CTC AAG CCA GGC CAA CCA ATG TAC
320 340 360

End
TGA AAAATGACCCTAAACTACTTCTAAACCCTCTCGATTCTTTTCACGTTTCATACAACACCTAGTTTTATTTATTTTC
380 400 420

TTTTCAATCTGAGTAGTTGAGTTTCGATCACTCACATAGAACTATTTTTT6CCATTTAAATAAAGTATTCTCTCAAAT
440 460 480 500

GATGCGATACTATAATACTCTTTGCCATATATTACATTCATTCAAATAGGCTATGTTTCTATATCCGTTTCCGATTCT
520 540 560 580

TGTCTGCAAGCAAGGTTCCCTATCATTACCGATTGTTCACTATGGTTGGAGCTC
600 620 640

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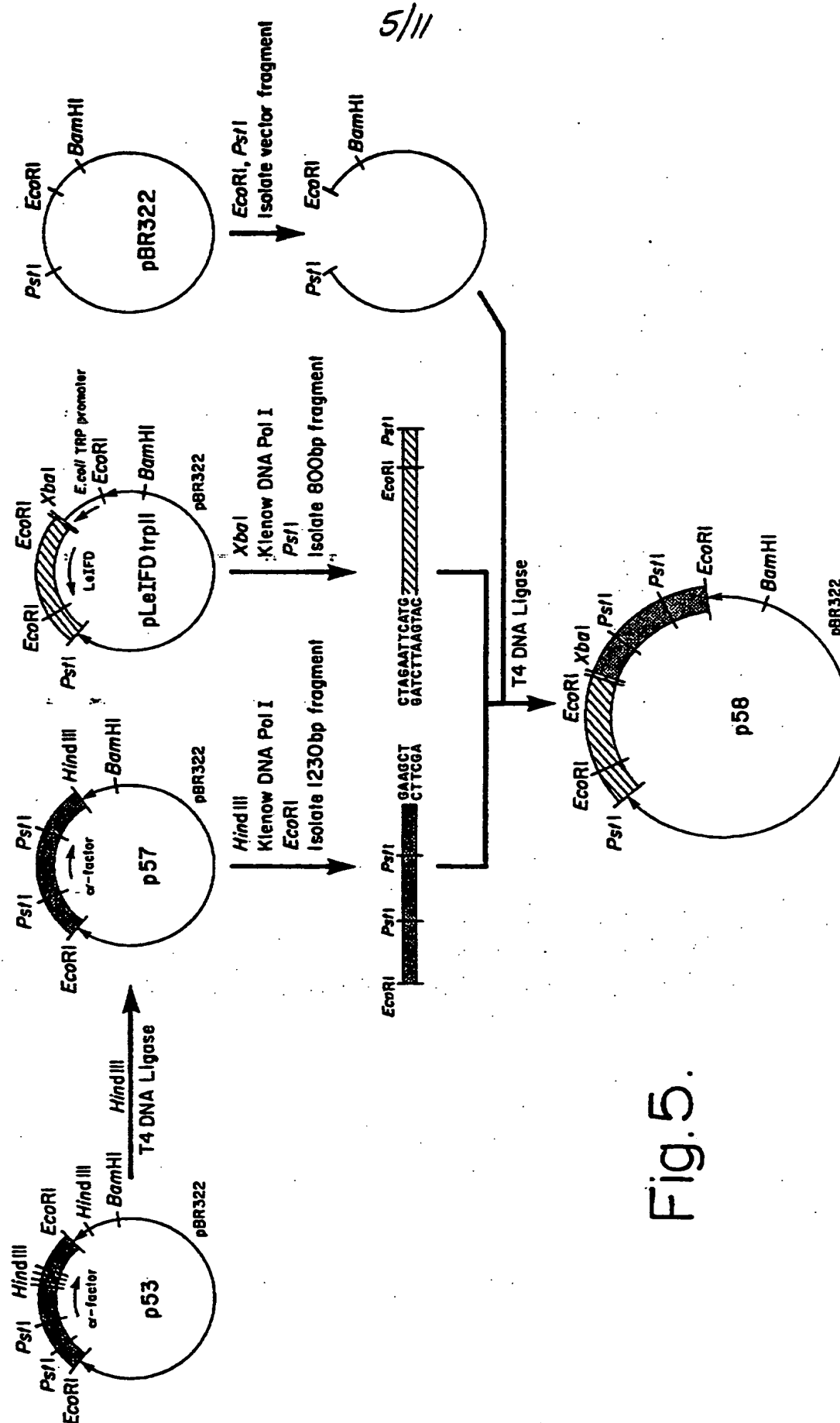


Fig. 5.

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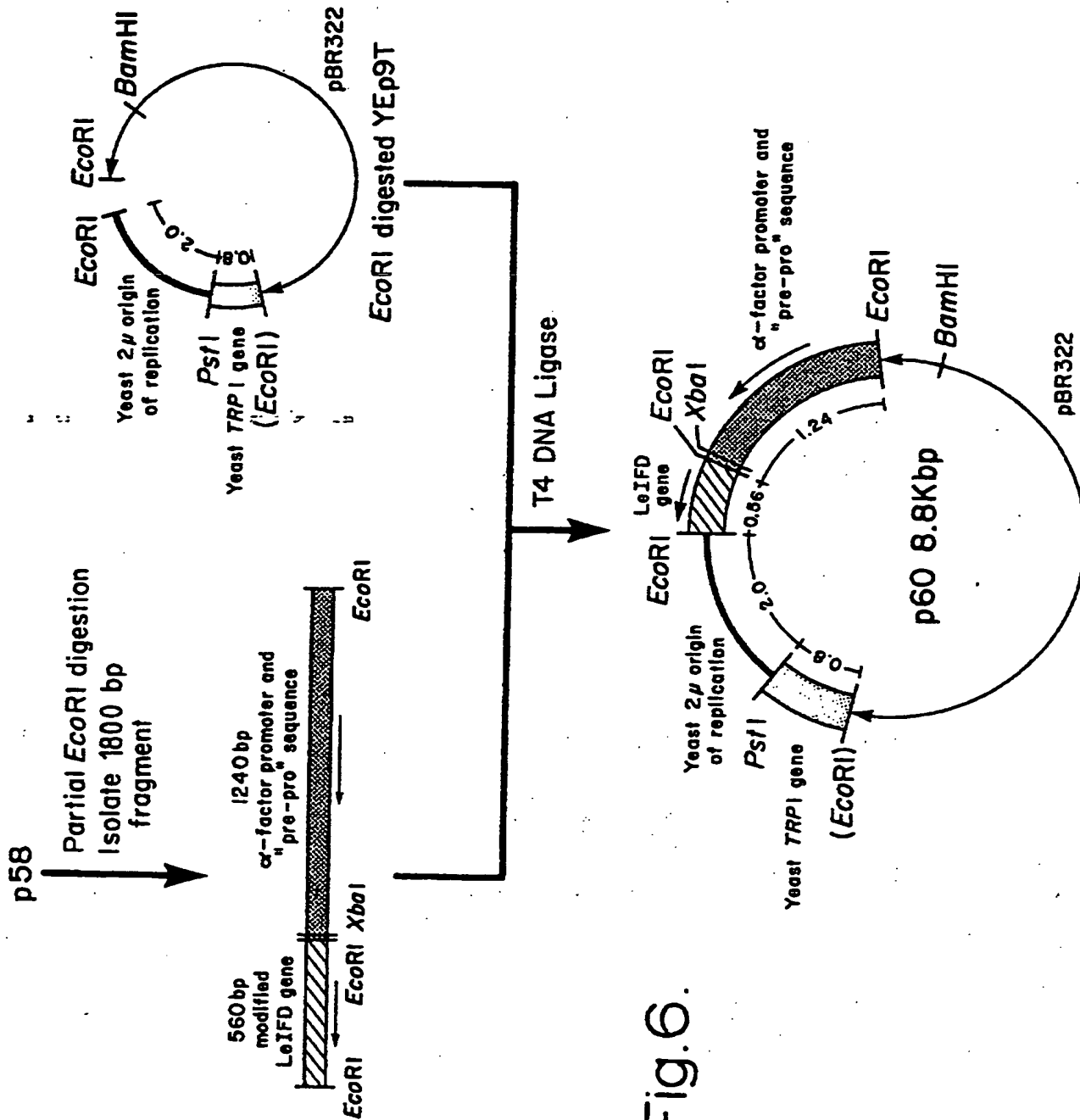
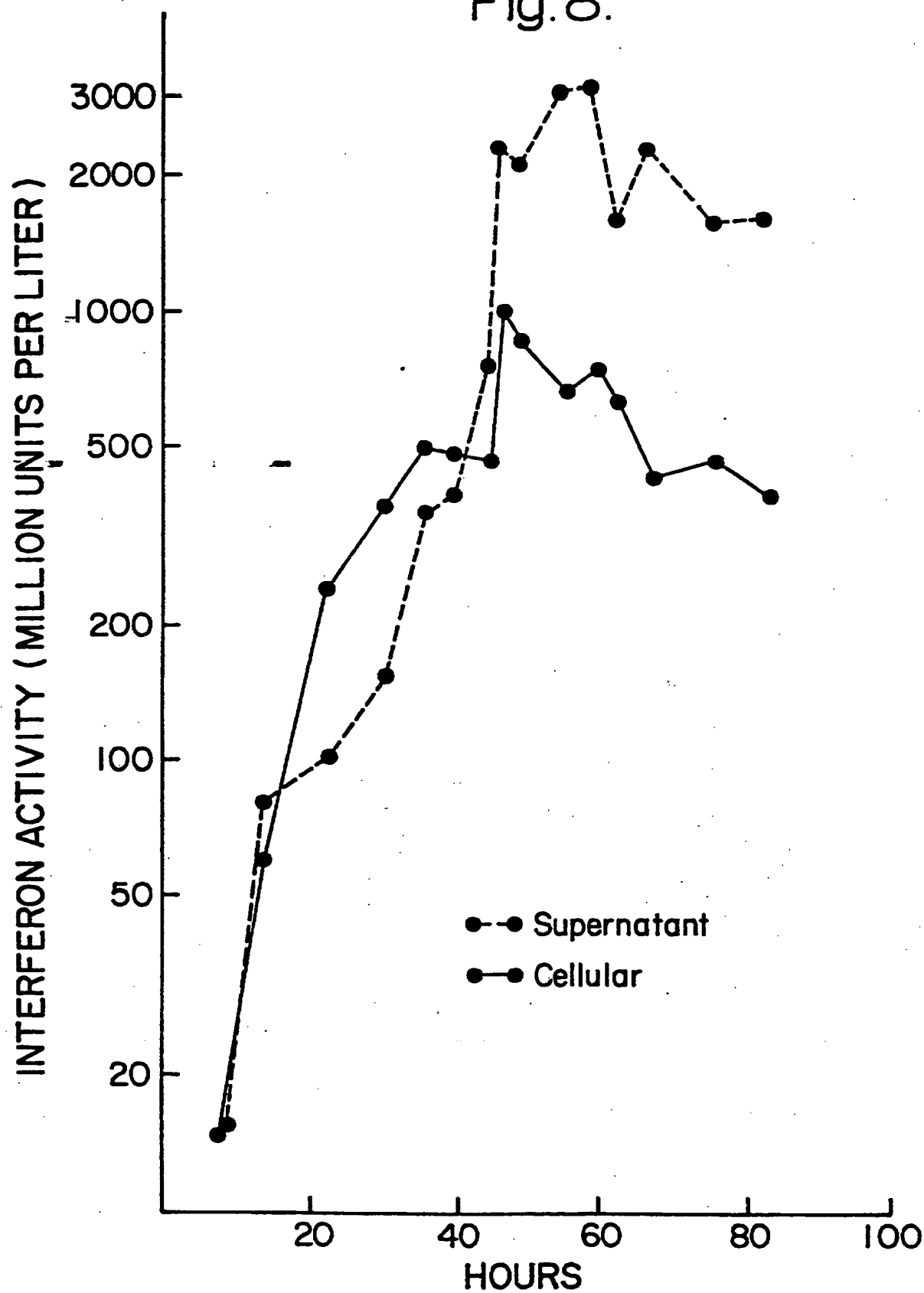


Fig.6.

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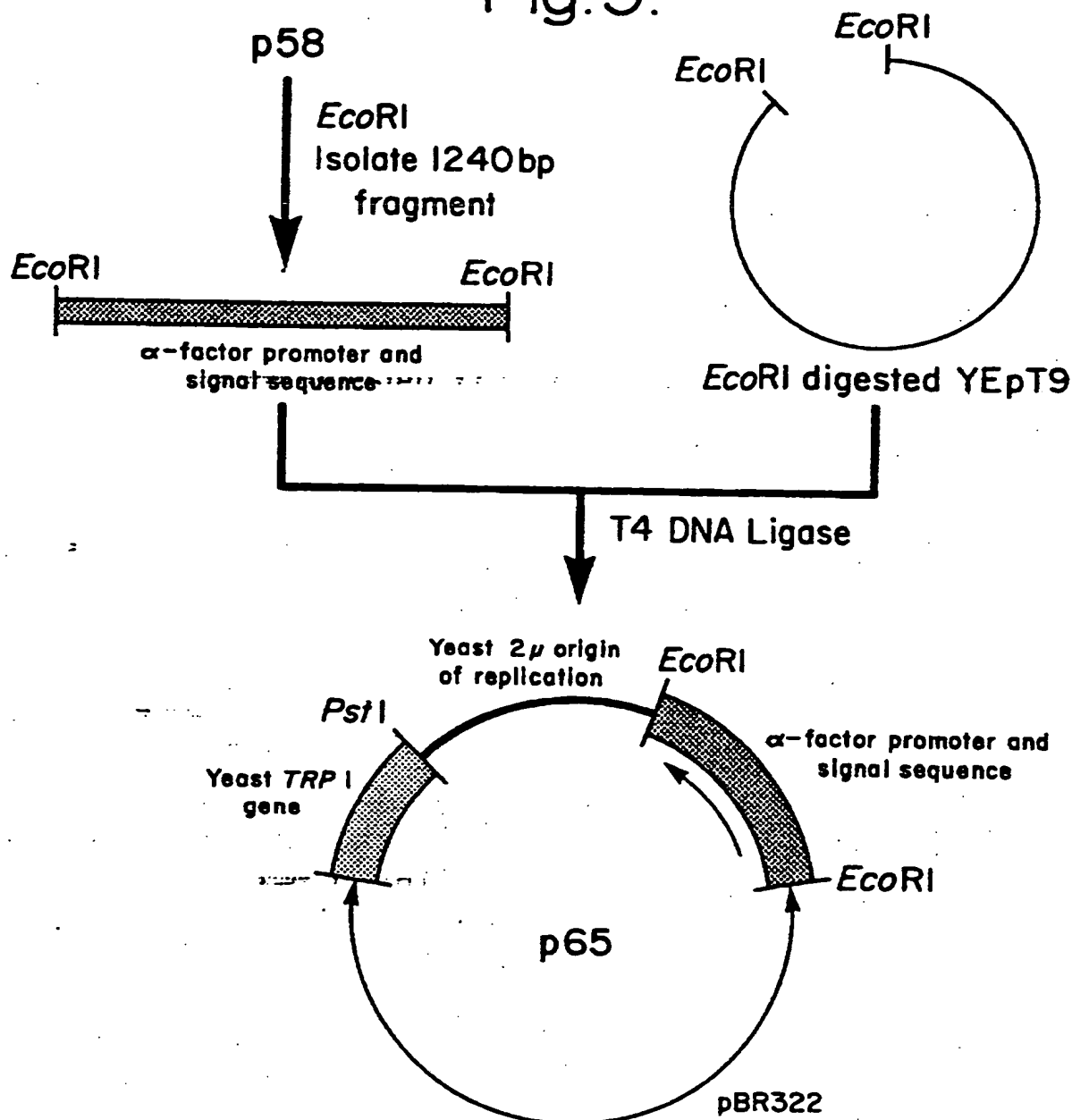
Fig.8.



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Fig.9.



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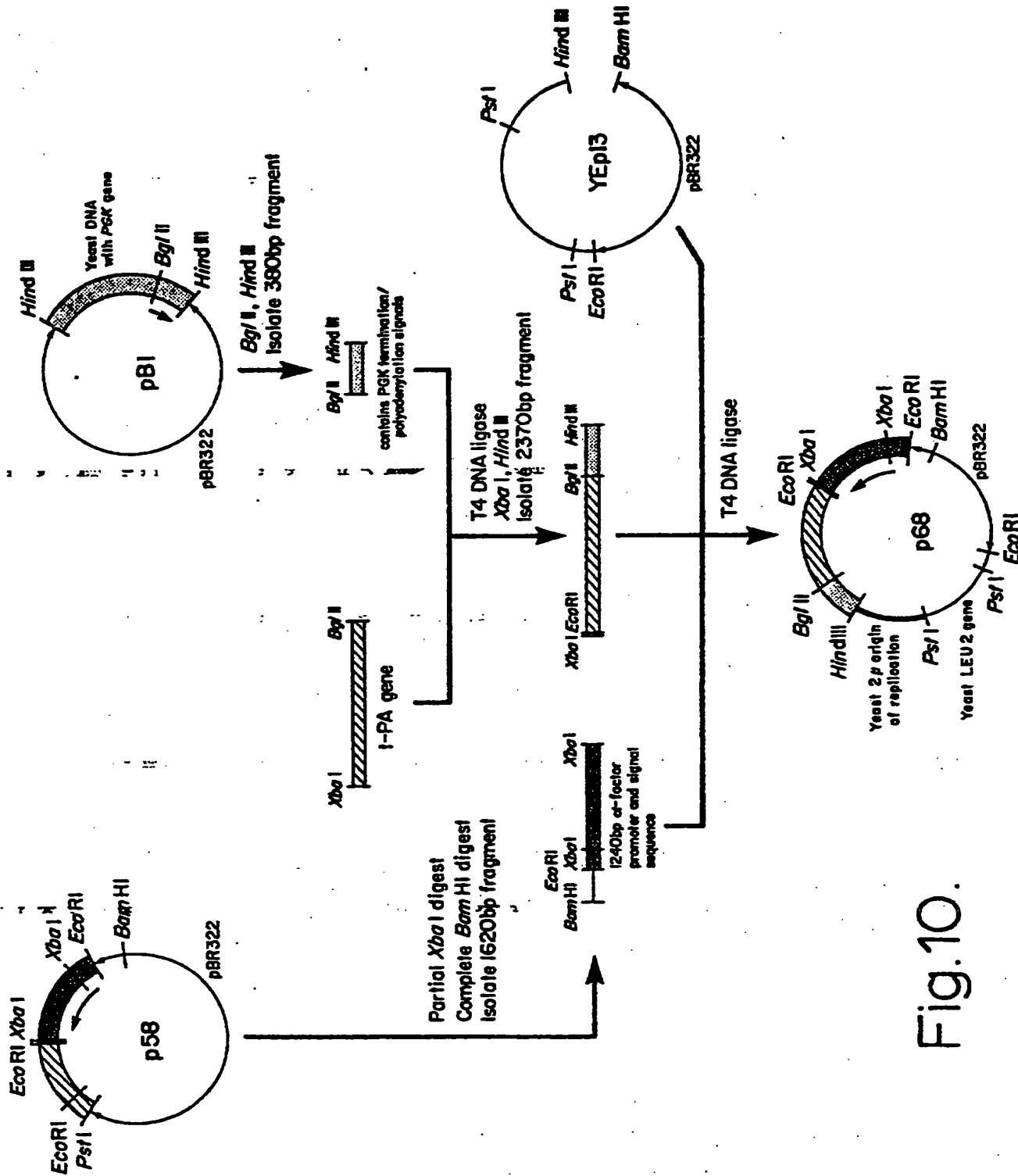


Fig.10.

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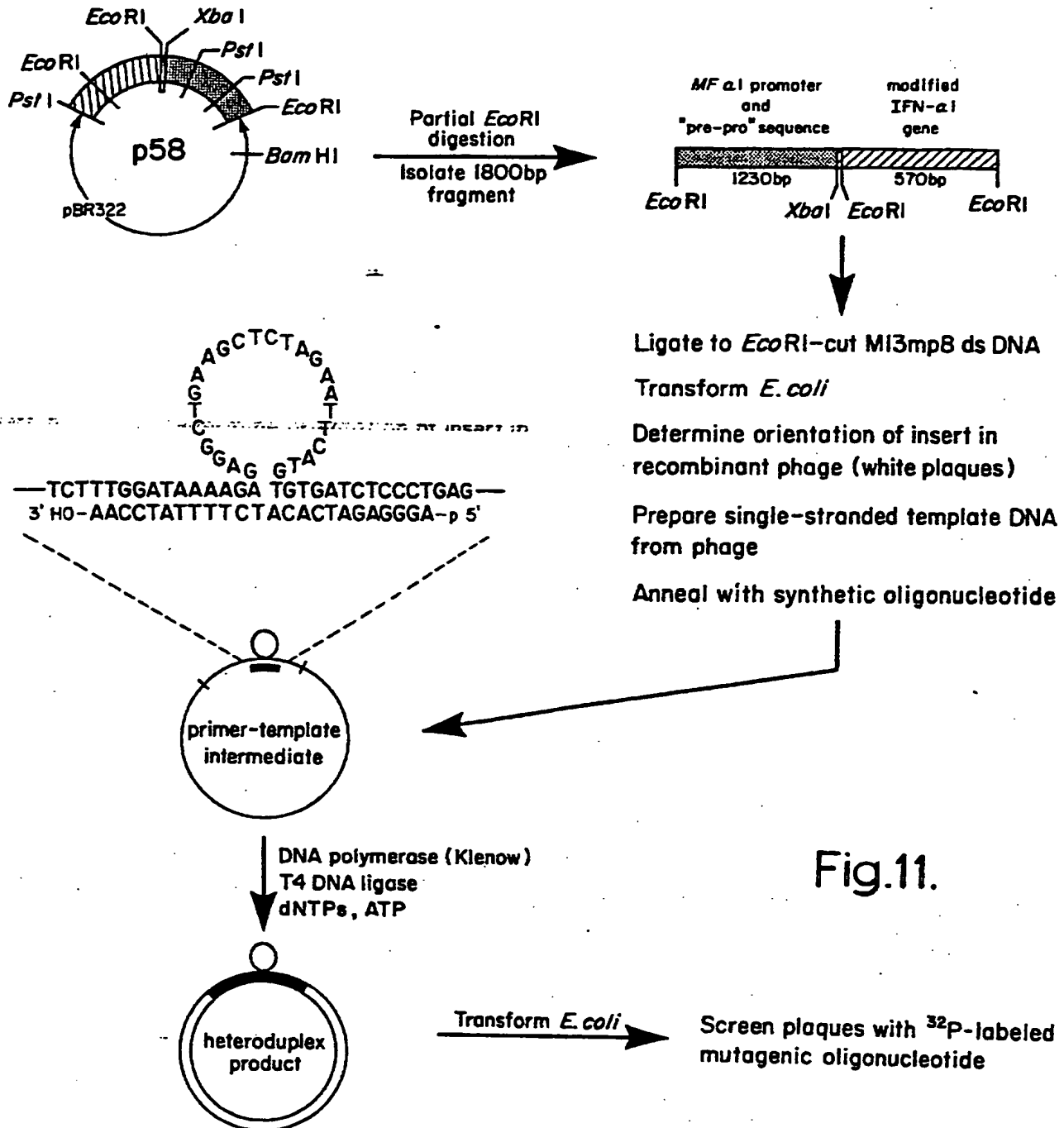


Fig.11.

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1	10	20
MFa1	MetArgPheProSerIlePheThrAlaValLeuPheAlaAlaSerSerAlaLeuAlaAla	
MFa2	MetLysPheIleSerThrPheLeuThrPhe-----IleLeuAlaAla	
Consensus	Met---Phe---Ser---Phe-----LeuAlaAla	
MFa1	30	40
MFa2	ProValAsnThr-----ThrThrGluAspGluThrAlaGlnIleProAlaGluAlaValIle	
Consensus	ValSerValThrAlaSerSerAspGluAspIleAlaGlnValProAlaGluAlaIleIle	
	-----Thr-----AlaGln-----ProAlaGluAla-----Ile	
MFa1	50	60
MFa2	GlyTyrLeuAspLeuGluGlyAspPheAspValAlaValLeuProPheSerAsnSerThr	
Consensus	GlyTyrLeuAspPheGlyGlyAspHisaspIleAlaPheLeuProPheSerAsnAlaThr	
	GlyTyrLeuAsp-----GlyAsp-----Ala-----LeuProPheSerAsn-----Thr	
MFa1	70	80
MFa2	AsnAsnGlyLeuleuPheIleAsnThrThrIleAlaSerIleAlaAlaLysGluGluGly	
Consensus	AlaSerGlyLeuleuPheIleAsnThrThrIleAlaGluAlaAlaGluLysGluGlnAsn	
	-----GlyLeuleuPheIleAsnThrThrIleAla-----Ala-----LysGlu-----	
MFa1	90	100
MFa2	ValSerLeuAspLysArgGluAlaGlu-----AlaTrpHisTrpLeuGlnLeuLysPro	
Consensus	ThrThrLeuAlaLysArgGluAlaValAlaAspAlaTrpHisTrpLeuAsnLeuArgPro	
	-----Leu-----LysArgGluAla-----AlaTrpHisTrpLeu-----Leu-----Pro	
MFa1	110	120
MFa2	GlyGlnProMetTyrLysArgGluAlaGluAlaGluAlaTrpHisTrpLeuGlnLeuLys	
Consensus	GlyGlnProMetTyrLysArgGluAlaAsnAlaAspAlaTrpHisTrpLeuGlnLeuLys	
	GlyGlnProMetTyrLysArgGluAla-----Ala-----AlaTrpHisTrpLeuGlnLeuLys	
MFa1	130	140
MFa2	ProGlyGlnProMetTyrLysArgGluAlaAspAlaGluAlaTrpHisTrpLeuGlnLeu	
Consensus	ProGlyGlnProMetTyr-----	
	ProGlyGlnProMetTyr-----	
MFa1	150	160
Consensus	LysProGlyGlnProMetTyrLysArgGluAlaAspAlaGluAlaTrpHisTrpLeuGln	

MFa1	168	
Consensus	LeuLysProGlyGlnProMetTyr-----	

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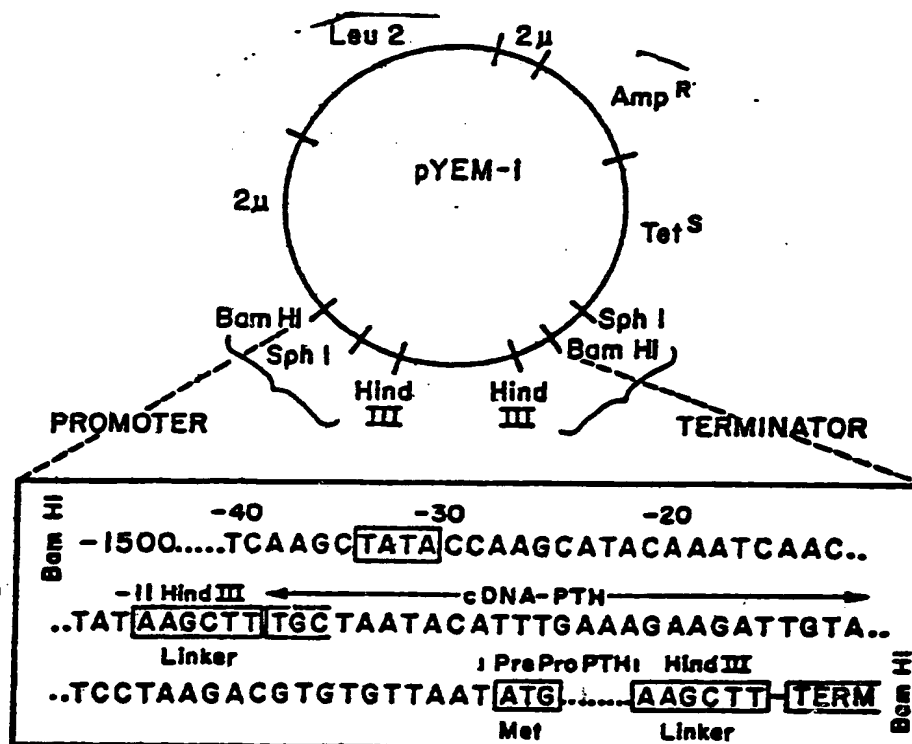
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(54) Title: PRODUCTION OF MATURE PROTEINS IN TRANSFORMED YEAST

(57) Abstract

A method for producing a mature protein in yeast transformed to express a corresponding precursor, wherein the mature protein sequence is contained in the precursor and is flanked proximally or both proximally and distally by a pair or triplet of basic amino acid residues. The method comprises proteolytic processing by an endopeptidase and exopeptidase present in the yeast. Yeast transformed by a plasmid containing a cDNA sequence encoding bovine preproparathyroid hormone is also disclosed.



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PRODUCTION OF MATURE PROTEINS
IN TRANSFORMED YEAST

BACKGROUND OF THE INVENTION

1. Field of the Invention.

5 This invention relates to a method for producing a mature protein in transformed yeast and further relates to Saccharomyces cerevisiae transformed by a plasmid containing a preproparathyroid hormone cDNA insert.

10 2. Description of the Prior Art.

 Recombinant DNA technology now makes it possible to isolate specific genes or portions thereof from higher organisms, such as man and other animals, and to transfer the genes or fragments
15 to a microorganism species, such as E. coli or yeast. The transferred gene is replicated and propagated as the transformed microorganism may become endowed with the capacity to make whatever protein the gene or fragment encodes, whether it
20 be an enzyme, a hormone, an antigen or an antibody, or a portion thereof. The microorganism passes on this capability to its progeny, so that in effect, the transfer results in a new strain, having the described capability.

25 Recombinant DNA conventionally utilizes transfer vectors. A transfer vector is a DNA molecule which contains genetic information which insures its own replication when transferred to a host microorganism strain. Plasmids are an
30 example of a transfer vector commonly used in genetics. Although plasmids have been used as the

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transfer vectors for the work described herein, it will be understood that other types of transfer vectors may be employed. Plasmid is the term applied to any autonomously replicating DNA unit which might be found in a microbial cell, other than the genome of the host cell itself. A plasmid is not usually genetically linked to the chromosome of the host cell. Plasmid DNA exists as doublestranded ring structures generally on the order of a few million daltons molecular weight, although some are greater than 10^6 daltons in molecular weight. They usually represent only a small percent of the total DNA of the cell. Transfer vector DNA is usually separable from host cell DNA by virtue of the great difference in size between them. Transfer vectors carry genetic information enabling them to replicate within the host cell.

Plasmid DNA exists as a closed ring. However, by appropriate techniques, the ring may be opened, a fragment of heterologous DNA inserted, and the ring reclosed, forming an enlarged molecule containing the inserted DNA segment.

Transfer is accomplished by a process known as transformation. During transformation, host cells mixed with plasmid DNA incorporate entire plasmid molecules into the cells. Once a cell has incorporated a plasmid, the latter is replicated within the cell and the plasmid replicas are distributed to the progeny cells when the cell divides.

Genetic information contained in the nucleotide sequence of the plasmid DNA, including heterologous DNA inserted into the plasmid, can in principle be expressed in the host cell. The inserted heterologous DNA typically representing

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a single gene, is expressed when the protein product coded by the gene is synthesized by the organism.

Once a gene has been isolated, purified and inserted into a plasmid or other vector, the availability of the gene in substantial quantity is assured. After transfer of the vector into a suitable microorganism, the gene replicates as the microorganism proliferates. The vector containing the gene is easily purified from cultures of the host microorganism by known techniques and separable from the vector by restriction endonuclease cleavage followed by gel electrophoresis. The protein product expressed by the heterologous gene can also be recovered in substantial quantities from cultures of the host microorganism by harvesting the culture and retrieving the protein product contained in the harvested cells. (For further detail of recombinant DNA technology, and an explicit exposition of the utility of producing proteins such as hormones, etc., by recombinant DNA technology, see U.S. Patent No. 4,237,224, issued December 2, 1980 to Cohen et al., and U.S. Patent No. 4,322,499, issued March 30, 1982 to Baxter et al. Patents and articles cited herein are incorporated by reference wherever such citations occur and shall be considered incorporated in their entirety as if set forth in full)..

Recombinant DNA thus holds great promise for economically producing substantial quantities of useful proteins that are difficult or costly to isolate in such quantities from mammalian tissue. A major and nearly universal problem in producing useful proteins, however, is the construction of the actual genetic material to be inserted into the transfer vector.



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Conventional means provide for enzymatically preparing desired genetic material by reverse transcription. Mature messenger RNA (mRNA), which is chemically similar to DNA and retains most of the information coded in DNA, can be extracted from tissue in which the desired gene is active. mRNA is separated from other RNA material in the tissue and complementary DNA (cDNA) is produced by the enzyme reverse transcriptase, and at times polymerase I for the synthesis of the second strand. This cDNA, a complementary copy of mRNA and similarly containing the information coded in RNA, is often further altered in known ways to be suitable for insertion into a plasmid vector.

(See W. Mahoney & S. Henikoff, Univ. of Washington Medicine, Vol. 8, No. 4, pp. 6-14 (Winter, 1981)).

cDNA enzymatically prepared by reverse transcription has the potential to express a protein chain identical to the protein expressed by tissue from which the mRNA was extracted. This alone is not sufficient, however, for the expression of desired mature animal proteins because many animal proteins, represented by such diverse classes as hormones, binding proteins, enzymes, antibodies, and collagen, are produced in nature in the form of larger precursors that are subsequently modified by cleavage to smaller bioactive forms commonly designated mature proteins. Thus, expression of cDNA synthesized by reverse transcription only has the potential to express the precursor of the mature protein product.

It has been known for several years that bacteria such as E. coli can remove the "pre" portion of its own secreted proteins. Examples include the processing of pre-ribose binding

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protein, pre-galactose binding protein and pre-arabinose binding protein. (L. Randall, et al., Eur. J. Biochem., Vol. 92, pp. 411-415 (1978); L. Randall, S. Hardy, and L. Josefsson, Proc. Natl. Acad. Sci. USA, Vol. 75, pp. 1209-1212 (1978)).

S. Chan, et al., Proc. Natl. Acad. Sci. USA, Vol. 78, pp. 5401-5405 (1981) has exploited the ability of E. coli to remove the "pre" sequence. Chan, et al., modified cDNA for human preproinsulin to encode a hybrid "pre" sequence containing portions of E. coli and mammalian "pre" sequence. E. coli expressed the hybrid protein and correctly removed the "pre" sequence by intra-cellular processing. Thus, Chan, et al., was able to modify human preproinsulin cDNA in a way that would allow E. coli to produce proinsulin.

It is also known that yeast shares the ability to remove "pre" sequences from its own pre-proteins. Furthermore, when an E. coli preprotein was genetically engineered into yeast, pre-B-lactamase was processed to B-lactamase. (Roggenkamp, et al., Proc. Natl. Acad. Sci. USA, Vol. 78, No. 7, pp. 4466-4470 (1981)).

The above type of processing of preproteins, however, will not process to mature proteins many of the mammalian hormone precursors and many of the other interesting mammalian protein precursors in E. coli. These latter hormone and protein precursors contain a "pro" portion which is not processed by the enzymatic mechanism responsible for processing the "pre" portion of preproteins. As shown above, for example, the natural precursor for insulin, i.e. preproinsulin is processed in E. coli to form proinsulin.

Many investigators have been unable to express pre-proteins in yeast or E. coli, let



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alone get processing. Expensive and time consuming, investigative efforts have focused almost exclusively on genetically eliminating the "pre" sequences and the "pro" sequences in attempting to express mature proteins without intermediates.

In several prior art approaches, the need for processing precursor proteins has been overcome. Insulin is the result of natural processing in human tissue involving cleaving two peptide chains, A and B, from the single large precursor preproinsulin and assembling the A and B chains to form the mature hormone insulin. The A and B chains are located within proinsulin and hence E. coli which processes preproinsulin to proinsulin does not produce the mature hormone insulin. An approach to obtaining mature insulin using E. coli employs chemically synthesized genes compatible with E. coli.

A double-stranded synthetic DNA-coding sequence for the insulin A chain was synthesized chemically from fundamental nucleotide units to yield the correct coding sequence. An extra amino acid (methionine) was added at one end. This end was fused to the bacterial gene for the enzyme B-galactosidase which results in accumulations of fused B-galactosidase-insulin-A-chain protein. This same procedure was repeated for the B-chain which resulted in the production of fused B-galactosidase-insulin-B-chain protein.

The fused proteins are insoluble in water and readily isolated from broken cells. The A and B chains of insulin are released from B-galactosidase at the extra methionine by cyanogen bromide cleavage and subsequently mixed together under conditions that allow formation of disulfide bonds between A

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and B chains, yielding mature insulin. (W. Miller, J. of Pediatrics, Vol. 99, pp. 1-15 (1981); D. Goeddel, et al., Proc. Natl. Acad. Sci. USA, Vol 76, pp. 106-110 (1979)).

- 5 The above prior art approach overcomes the need for processing a precursor protein, but in turn requires processing of the fused B-galactosidase-insulin-A-chain and B-galactosidase-insulin-B-chain proteins to mature insulin.
- 10 Moreover, chemical synthesis of the DNA coding sequences for A-chain and B-chain involves substantial costs, even when considering that the B-galactosidase-insulin-A-chain gene and B-galactosidase-insulin-B-chain gene after being
- 15 synthesized are easily replicated for subsequent production of insulin. (D. Williams, et al., Science, Vol. 215, pp. 687-689 (Feb. 1982); W. Mahoney, Univ. of Wash. Medicine, supra).

- The approach of chemically synthesizing DNA
- 20 encoding for mature proteins has also been shown to be effective for bacterial production of human somatostation. (K. Itakura, et al., Science, Vol. 198, pp. 1056-1063 (1977)). However, insulin chains A and B and human somatostation are
- 25 relatively small sequences and chemically synthesized DNA coded for them are relatively small. In the case of larger proteins, chemical synthesis of the DNA coding sequence coded for such proteins is prohibitively time consuming.

- 30 One prior art approach, now often followed, utilizes chemically synthesized DNA in conjunction with enzymatically prepared cDNA to produce a gene which instructs production of mature hormone in bacteria. Human growth hormone (HGH) is a
- 35 protein of 191 amino acids, its precursor having an additional 26 amino acid "pre" portion. cDNA

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encoding the precursor was enzymatically prepared from mRNA isolated from human pituitary tissue. The first useful cleavage site of the cDNA occurs at the site encoding amino acid residues 23-24 of HGH. Treatment of the cDNA with restriction endonuclease Hae III gives a DNA fragment of 551 base pairs which includes coding sequences for amino acids 24-191 of HGH. A gene fragment having coding sequences for residues 1-23 of HGH (and an initiation codon) was chemically synthesized. The two DNA fragments were combined to form a synthetic-natural hybrid gene which when inserted into a plasmid vector directed expression of mature HGH in *E. coli*. (D. Goeddel, et al., Nature, Vol. 281, pp. 544-548 (October 1979)).

Using a similar strategy of cleavage and reconstruction of DNA for the mature protein, R. Lawn et al., Nucleic Acids Research, Vol. 9, No. 22, pp. 6103-6114 (1981), expressed mature human albumin in *E. coli*.

This general approach, however, requires time consuming chemical synthesis of desired gene fragments, cleavage of cDNA assuming the availability of useful cleavage sites and difficult genetic construction of plasmids from DNA fragments. Furthermore, in both of the above examples, an initiator methionine was left at the NH₂-terminal. The initiator methionines cannot practically be removed since HGH and albumin also have methionines located elsewhere in the sequence. Thus, removing the initiator methionine by cyanogen bromide cleavage, would result in cleavage at the other methionines. This would result in a protein split into cleaved fragments. Both the HGH and albumin produced by the above approach are "mature" proteins which start with methionines. Hence they

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are not "real" mature proteins.

The prior art approaches set forth above illustrate that a major and nearly universal problem in producing mature proteins is the construction of the actual genetic material to be inserted into transfer vectors. Procedures exist for preparing cDNA from mRNA isolated from mammalian or other higher order animal tissue, but mammalian and higher order animal proteins are most often expressed as precursors and subsequently processed into the mature protein in cells of origin. The prior art has identified E. coli and yeast as microorganisms capable of processing precursors containing the "pre" portion, but this class of precursors excludes many of precursors of interest. The prior art thus has not identified a microorganism suitable for cloning mammalian and higher order animal genes which is capable of processing to mature proteins precursors of greatest interest. The prior art approaches attempt to solve the problem by constructing genes that code for mature protein. However, although procedures now exist for identifying nucleotide coding sequences for mature proteins, chemical synthesis of DNA sequences encoding mature proteins or fragments thereof for use in hybrid genes is costly and time consuming, often prohibitively so.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows inferred protein cleavage sites within the precursor of yeast α -factor, where "K" designates lysine and "R" designates arginine amino acid residues.

FIG. 2 shows the cDNA sequence encoding preproparathyroid hormone and the unique Pvu II and Hinf I cleavage sites.

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FIG. 3 shows certain portions of the nucleotide sequence of the pYEM-1 plasmid.

SUMMARY OF THE INVENTION

In the present invention, a method is disclosed for producing protein in yeast transformed to express a corresponding precursor containing a pair or triplet of basic amino acid residues located proximally and/or distally adjacent to the protein portion of the precursor sequence comprising proteolytic processing by the yeast of the precursor at the site of such pairs or triplets of basic amino acid residues. The method comprises proteolytic processing by transformed yeast which contains an endopeptidase, designated herein as a trypsin-like enzyme or enzymes. The trypsin-like enzyme or enzymes proteolytically process the precursor at the site of such pairs or triplets of basic amino acid residues by cleaving at the distal side of such pairs or triplets. The method further comprises proteolytic processing by transformed yeast that contains an exopeptidase, designated herein a carboxypeptidase-B-like enzyme or enzymes. The carboxypeptidase-B-like enzyme or enzymes proteolytically process the precursor at the site of such pairs or triplets of basic amino acid residues by degrading such pairs or triplets of basic amino acid residues remaining distally adjacent to the protein portion of the precursor sequence after the cleavage by the trypsin-like enzyme or enzymes.

In the present invention, the above method is further disclosed for proteolytic processing of proto-proteins to mature proteins. Proto-proteins, defined with greater specificity infra, consist generally of precursor proteins in which the

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protein portion of the precursor sequence is identical in structure to the mature protein except for the absence of the amino terminal and the carboxyl terminal in the precursor sequence. The above method is also disclosed for proteolytic processing of certain non-proto-proteins. For example, the above method is disclosed for proteolytic processing of preproinsulin or proinsulin to mature insulin. The above method is disclosed for producing mammalian insulin generally as well as human, bovine, and porcine insulin specifically. According to the method, preprocalcitonin and procalcitonin may be proteolytically processed by transformed yeast to form mature calcitonin or a calcitonin relative in the case of animal calcitonin generally and human, bovine, and porcine calcitonin specifically.

In the present invention, a recombinant DNA plasmid transfer vector useful for transforming yeast comprising a DNA sequence comprising the preproparathyroid gene cDNA sequence is disclosed as well as the plasmid pYEM-1 and yeast transformed by a plasmid comprising the above transfer vector and yeast transformed by the plasmid pYEM-1.

DESCRIPTION OF THE SPECIFIC EMBODIMENT

Proto-proteins may consist of precursors for which DNA and mRNA encoding the precursors naturally occur in animals. This type of proto-protein is designated source natural proto-proteins. Proto-proteins may also consist of precursors in which synthetic DNA encodes the precursor. This type of proto-protein is designated source synthetic proto-protein. For example, by chemical synthesis, or alternatively by enzymatic cleavage, rearrangement and subsequent fusion, DNA can be synthesized so that the precursor which

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it encodes has the cleavage properties discussed below. Production of mature protein might be enhanced by transforming yeast with synthetic DNA encoded for a precursor having repetitive sequences of the mature protein, each sequence being flanked by appropriate cleavage sites.

Source natural proto-proteins are illustrated by, but not limited to, certain hormone precursors, including preproparathyroid (J. Habener & J. Potts, The New England Journal of Medicine (Second Part), Vol. 299, No. 12, pp. 635-643 (Sept. 1978)), preprosomatostatin (P. Hobart, et al., Nature, Vol. 288, pp. 137-139 (November 1980)), AVP-NpII precursor to arginine vasopressin and its corresponding neurophysin (H. Land, et al., Nature, Vol. 295, pp. 299-303 (January 1982)), corticotropin B-lipotropin precursor to corticotropin (ACTH) and B-lipotropin (B-LPH) (S. Nakanishi, et al., Nature, Vol. 278, pp. 423-427 (March 1979)), preproglucagon (P. Lund, et al., Proc. Natl. Acad. Sci. USA, Vol. 79, pp. 345-349 (January 1982)), and pro-opiomelanocortin (POMC) precursor to B-endorphin and Met- and Leu-enkephalin precursor (M. Comb, et al., Nature, Vol. 295, pp. 663-666, (February 1982)).

Source natural proto-proteins are also illustrated by melittin precursor (G. Suchanek, et al., Eur. J. Biochemistry, Vol. 60, pp. 309-315 (1975); G. Suchanek, et al., Proc. Natl. Acad. Sci. USA, Vol. 75, pp. 701-704 (1978)) and serum albumin precursors (R. Lawn, et al., Nucleic Acids Research, Vol 9, No.22, pp. 6103-6114 (1981)).

As reported in the above citations, these precursors contain within their sequence at least one mature protein sequence. Where there is a single mature protein sequence contained in the precursor it is flanked proximally by a pair or

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triplet of basic amino acid residues consisting of lysine and/or arginine and is flanked distally by either the carboxyl-terminal of the precursor or a pair or triplet of basic amino acid residues lysine and/or arginine. If there are several mature protein sequences contained in the precursor, at least one of the mature protein sequences is flanked proximally by a pair or triplet of such basic amino acid residues and is flanked distally by either the carboxyl-terminal of the precursor or a pair or triplet of such basic amino acid residues. Any precursor protein falling within this description is defined herein as a proto-protein, whether it be source natural or source synthetic.

As reported in the above citations in connection with observing the production of mature proteins in mammals and other higher order animals, the cleavage site located on the distal side of a pair or triplet of such basic amino acid residues is readily attacked by endopeptidases with trypsin-like activity. After endopeptidase cleavage, any residual basic residues remaining adjacent to and on the distal side of the mature protein are susceptible to degrading, i.e. selective removal, by exopeptidases with activity resembling that of carboxypeptidase-B.

Thus, for example, in preproparathyroid hormone the mature protein is flanked proximally by the basic triplet lysine-lysine-arginine and is flanked distally by the carboxyl-terminal of the precursor. A single cleavage by a trypsin-like enzyme is sufficient to produce the mature hormone. In other proteins such as the glucagon precursor, two mature glucagon proteins are flanked both proximally and distally by a basic pair lysine-arginine. Combined cleavage by a trypsin-like

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enzyme and degradation of the resulting carboxyl-terminal by a carboxypeptidase-B-like enzyme are required to produce the mature proteins.

5 The method of the present invention comprises preteolytic processing by yeast of proto-proteins to mature proteins. In the method, transformed yeast naturally containing a trypsin-like enzyme or enzymes and a carboxypeptidase-B-like enzyme or enzymes, proteolytically release mature proteins
10 from larger precursors. These enzymes will effectively cleave and degrade proto-proteins to mature proteins. This is confirmed by a trypsin-like cleavage, discussed infra, of preproparathyroid hormone yielding mature parathyroid hormone. This is
15 further confirmed by yeast processing its own mating factor, α -factor. (T. Tanaka, et al., J. Biochemistry, Vol. 82, pp. 1681-1687 (1977)). As shown in FIG. 1, the nucleotide sequence of α -factor shows that yeast naturally expresses a
20 precursor containing four distinct codings for mature α -factor. Three of the four α -factors in the precursor are flanked distally by a pair of basic amino acids residues. A trypsin-like cleavage in combination with a carboxypeptidase-
25 B-like degrading naturally yields correctly processed C-termini for these three α -factors. After a trypsin-like cleavage, N-termini of the four α -factors are flanked proximally by a series of several glutamic acid and alanine amino acid
30 residues. These latter residues are in turn removed by an aminopeptidase. The foregoing natural endopeptidase and exopeptidase activity in yeast in combination with the virtual uniform presence of pairs and triplets of lysine and/or arginine
35 flanking mature hormone sequences in proto-proteins underlies the present invention.

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Although preproinsulin and proinsulin containing disulfide bonds are not proto-proteins as defined herein they will nevertheless undergo proteolytic processing in yeast transformed to express the preproinsulin or proinsulin. A pair or triplet of basic amino acid residues are located distally and/or proximally adjacent to the insulin-A-chain and the insulin-B-chain portions of the sequence which constitute the protein portion of the precursor preproinsulin and proinsulin sequence. The requisite disulfide bonds between the insulin-A-chain portion of the sequence and the insulin-B-chain portion of the sequence will be formed in yeast. (cf. the numerous examples of disulfide bond formation in yeast disclosed in M. Dayhoff, Atlas of Protein Sequence and Structure, Vol. 5 and Supplements 1, 2 & 3 (National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C. 20007 (1972, 1973, 1976, and 1981))). Proteolytic processing at the site of such pairs or triplets of basic amino acid residues will yield mature insulin from preproinsulin or proinsulin containing the disulfide bonds.

In the absence of disulfide bond formation between the insulin-A-chain portion of the sequence and the insulin-B-chain portion of the sequence, proteolytic processing will yield insulin-A-chain and insulin-B-chain, which may be caused in turn to attach to one another by disulfide bonds by conventional means to form mature insulin. In this case, the insulin-A-chain and insulin-B-chain may be considered mature proteins and preproinsulin and proinsulin without disulfide bonds may be considered a proto-protein according to the above discussion of proto-proteins.

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Mature calcitonin contains disulfide bonds between the cysteines located at positions 1 and 7 of the sequence, contains a carbohydrate attached at the sequence at position 3, and the proline at position 32 has been amidated to pro-amide while the glycine at position 33 has been removed. Preprocalcitonin and procalcitonin will contain the requisite disulfide bonds. (cf. the numerous examples of disulfide bond formation in yeast as disclosed in Dayhoff, supra). A carbohydrate will be attached at position 3 in calcitonin. Preprocalcitonin and procalcitonin will undergo proteolytic processing in yeast transformed to express the preprocalcitonin or procalcitonin. A pair of basic amino residues are located proximally adjacent to the 33 amino acid sequence, while a triplet is located distally adjacent to the 33 amino acid sequence. It is expected that amidation of the proline located at 32 will occur in yeast after the cleavage distal to and degradation of the triplet. (cf. numerous examples of amidation in yeast as disclosed by Dayhoff, supra). In the event that a carbohydrate differing from the carbohydrate of mature calcitonin is formed by the yeast, the calcitonin relative containing the differing carbohydrate may be converted to mature calcitonin by conventional means. In the event that amidation following cleavage and degradation is suppressed, the calcitonin relative lacking the amidation may also be converted to mature calcitonin by conventional means.

By reverse transcription, cDNA can be prepared encoding any proto-protein of interest by isolating mRNA from tissues expressing the protein. Although many hormone and other protein genes have

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already been cloned in E. coli, yeast has heretofore not been the host of choice. cDNA not previously cloned in yeast can be rendered compatible with a yeast host by proper codon selection (J. Bennetzen & B. Hall, J. Bio Chem., Vol. 257, pp. 3026 (1982)) and by site specific mutagenesis of the cDNA (G. Simmons, et al., Nucleic Acid Research, Vol. 10, pp. 821 (1982)).

Thus, one of the fundamental problems with producing useful mature proteins by recombinant DNA techniques has been simplified in the case of mature proteins derived from proto-proteins. cDNA, although readily available for most proteins by reverse transcription of mRNA isolated from animal tissue, will express the precursor of the mature protein. Yeast, but not E. coli, has the requisite enzymes to process expressed proto-proteins, preproinsulin, or proinsulin to mature protein or insulin.

EXPERIMENTAL

In order to demonstrate the present invention, the following experiment was carried out.

The plasmid YEp-13 was obtained from Dr. Steven Henekoff, Fred Hutchinson, Dept. of Developmental Biology, Seattle, Washington, and can be constructed according to J. Broach, et al., Gene, Vol. 8, pp. 121-133, (1979). The gene which encodes yeast alcohol dehydrogenase 1 was modified according to Hitzelman, et al., Nature (London), Vol. 293, pp. 717-722 (1981), allowing the isolation of the transcription signals. These sequences, including the cloning site, were provided by Dr. G. Ammera. The plasmid YEp-13 was modified so that the tet^R gene of YEp-13 was interrupted at the Bam H1 site with the yeast alcohol dehydrogenase 1 gene promotor and RNA polymerase



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stop sequences. A Hind III site between the latter two elements provided the cloning site. These modifications of plasmid YEp-13 were accomplished by methods set forth generally in U.S. Patent 4,237,224, supra, and Methods of Enzymology, Vols. 65 and 68 wherein such methods are reviewed.

The cDNA sequence coding bovine preproparathyroid hormone, shown in FIG. 2 and further described in B. Kemper, et al., Hormonal Control of Calcium Metabolism (Ed. by D. Cohn, et al., published Excerpta Medica at Amsterdam, Oxford, and Princeton 1981) at pp. 19, was obtained from Dr. Byron Kemper, Department of Physiology and Biophysics and School of Basic Medical Sciences, University of Illinois-Urbana. This cDNA sequence was restricted with the enzymes PVU II and Hinf I at the sites shown in FIG.2. These enzymes were obtained from New England Biolaboratories, Beverly MA. The Hinf I site shown in FIG. 2 was filled with nucleotides using the enzyme DNA polymerase I (the large fragment) which was obtained from New England Nuclear, Boston, MA. This modified sequence was then blunt-end ligated to Hind III linkers and restricted with the enzyme Hind III. The Hind III linkers and Hind III enzyme were obtained from New England Biolaboratories, supra. The resulting DNA fragment was then ligated into the Hind III site of the modified plasmid YEp-13 forming a novel plasmid. This plasmid was designated pYEM-1. FIG. 3 shows certain portions of the nucleotide sequence of pYEM-1. The foregoing construction of pYEM-1 was accomplished by methods set forth generally in U.S. Patent No. 4,237,224, supra, the BLR M13 handbook, and Methods of Enzymology, Vols. 65 and 68 wherein such methods are

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reviewed.

After constructing pYEM-1, yeast cells were transformed with the plasmid using the methods of Beggs, Nature (London), Vol. 275, pp. 104-109 (1978) and Hinnen, et al., Proc. Natl. Acad. of Sci. USA, Vol. 75, pp. 1929-1933 (1978). Because pYEM-1 has the yeast leu 2 gene, the use of a leu 2 negative strain of yeast was used in the transformation for the purposes of selecting successful transformants. Yeast strain, X1069-2D, a strain of Saccharomyces cerevisiae defective in leu-2 function, was obtained from the Yeast Genetic Stock Center, Univ. of California-Berkeley.

Of course any other defective yeast strain, including strains within Saccharomyces pombe and other species, could be used. All that is required is that a complementation system be established between the yeast strain and the cloning/expression vector and that the vector be stably maintained in yeast. For example, a Trp 1 strain could be used if the Trp 1 gene was on the vector. To date, several stable transformation systems have been described. (A. Hinnen and B. Meyhack, Current Topics in Microbiology and Immunology, Vol. 96, pp. 101-117 (1981); C. Hollenberg, Current Topics in Microbiology and Immunology, Vol. 96, pp. 119-144 (1981)).

The transformed yeast cells containing plasmid pYEM-1 were grown in a leucine deficient media containing 5% glucose, yeast extract, yeast nitrogen base and other nutrients suitable for yeast strain X1069-2D. After 24 hours of growth at 30°C, the media was collected and the yeast cells lysed. Bioassay was performed according to conventional techniques and PTH



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radioimmunoassay was performed using Immuno Nuclear Corporation (Stillwater, MN) assays specific to the N-terminal, mid-molecule, and C-terminal regions of parathyroid hormone. The following table shows that both immunologically cross-reactive parathyroid hormone and biologically active parathyroid hormone is being produced in yeast.

TABLE

	PTH N- terminal RIA*	PTH Mid- molecule region RIA*	PTH C- terminal RIA*	Bioassay*
Cell lysate				
pYEM-1	16	16	16	10
control	0	0	0	0
Media				
pYEM-1	2	2	2	0.015
control	0	0	0	0

*expressed in nanomoles/ml

To confirm that correct processing had occurred, 50 ml of culture was prepared in which the parathyroid hormone producing yeast were grown in media containing ³⁵S methionine (80 µ ci/ml). After an overnight growth the cells were removed by centrifugation. The media was then incubated with specific N-terminal parathyroid hormone antibody. After two hours the antibody-antigen complex was recovered by centrifugation and washed three times with new media followed by an ether wash. This complex contained about 7,000 cpm of ³⁵S methionine incorporated into protein after TCA precipitation. This mixture was applied to a Beckman 890D

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sequencer according to the methods of Mahoney and Nute, Biochem. Vol. 19, pp. 4436 (1980) and subsequently degraded 40 cycles. Sequence analysis demonstrated that the ³⁵S methionine was all contained in cycles number 8 and 18. In mature PTH, methionine appears only at positions 8 and 18 in the sequence. If preproparathyroid hormone expressed by the yeast was left unprocessed, we would expect ³⁵S methionine in cycles 1, 2, 7, 11, 14, 49, and 59 reflecting the appearance of methionine at positions -31, -30, -25, -21, -18, +8, +18 in the preproparathyroid sequence.

The novel microorganism yeast strain X1069-2D transformed by novel plasmid pYEM-1, designated X1069-2D-pYEM-1, was placed on permanent deposit in the Northern Regional Research Center, U.S. Dept. of Agriculture, Peoria, Illinois 61604 on September 8, 1982. The NRRL number for X1069-2D-pYEM-1 is Y-15153. The plasmid pYEM-1 and the transfer vector contained therein may be removed from this novel yeast strain by known means.

While the invention has been described in connection with a specific embodiment thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations uses, or adaptations of the invention within the scope of the appended claims.



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CLAIMS

1. A method for producing protein in yeast transformed to express a corresponding precursor containing a pair or triplet of basic amino acid residues located proximally and/or distally adjacent to the protein portion of the precursor sequence, comprising proteolytic processing by the yeast of the precursor at the site of such pairs or triplets of basic amino acid residues.
2. The method of claim 1 wherein the proteolytic processing by yeast of the precursor at the site of such pairs or triplets of basic amino acid residues comprises cleaving, by a trypsin-like enzyme or enzymes present in the transformed yeast, at the distal side of such pairs or triplets of basic amino acid residues.
3. The method of claim 2 wherein the proteolytic processing by yeast of the precursor at the site of such pairs or triplets of basic amino acid residues further comprises degrading, by a carboxypeptidase-B-like enzyme or enzymes present in the transformed yeast, of any such pairs or triplets of basic amino acid residues remaining distally adjacent to the protein portion of the precursor sequence after the cleavage by the trypsin-like enzyme or enzymes.
4. The method of claim 1 wherein the corresponding precursor is a proto-protein.
5. The method of claim 4 wherein the proto-protein is source synthetic proto-protein.



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6. The method of claim 4 wherein the proto-protein is source natural proto-protein.
7. The method of claim 6 wherein the source natural proto-protein is bovine preproparathyroid hormone.
8. The method of claim 1 wherein the protein is mammalian insulin and the corresponding precursor is mammalian preproinsulin or proinsulin.
9. The method of claim 8 wherein the mammalian insulin and mammalian preproinsulin or mammalian proinsulin are members respectively of the group consisting of human insulin and human preproinsulin or human proinsulin, bovine insulin and bovine preproinsulin or bovine proinsulin, and porcine insulin and porcine preproinsulin or porcine proinsulin.
10. The method of claim 1 wherein the protein is animal calcitonin or an animal calcitonin relative and the precursor is animal preprocalcitonin or animal procalcitonin.
11. The method of claim 10 wherein the animal calcitonin or animal calcitonin relative and the animal preprocalcitonin or animal procalcitonin are members respectively of the group consisting of human calcitonin or human calcitonin relative and human preprocalcitonin or human procalcitonin, bovine calcitonin or bovine calcitonin relative and bovine preprocalcitonin or bovine procalcitonin, and porcine calcitonin or porcine calcitonin relative and porcine preprocalcitonin or pro-calcitonin.

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12. The method of claim 1 wherein the yeast is *Saccharomyces cerevisiae* or *Saccharomyces pombe*.

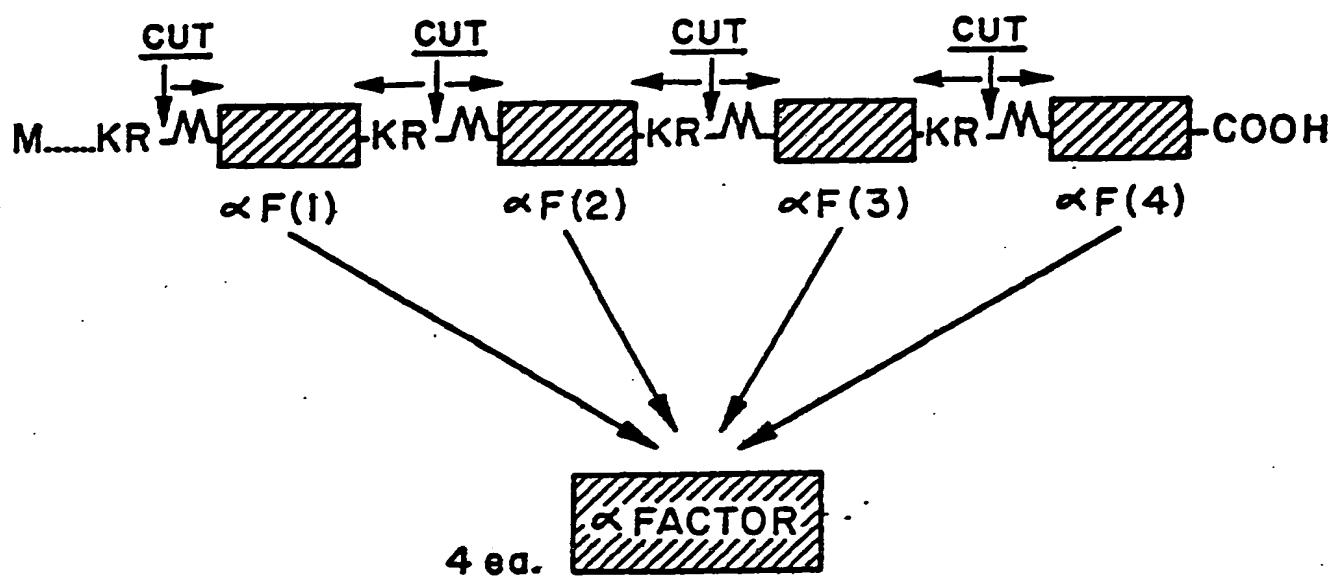
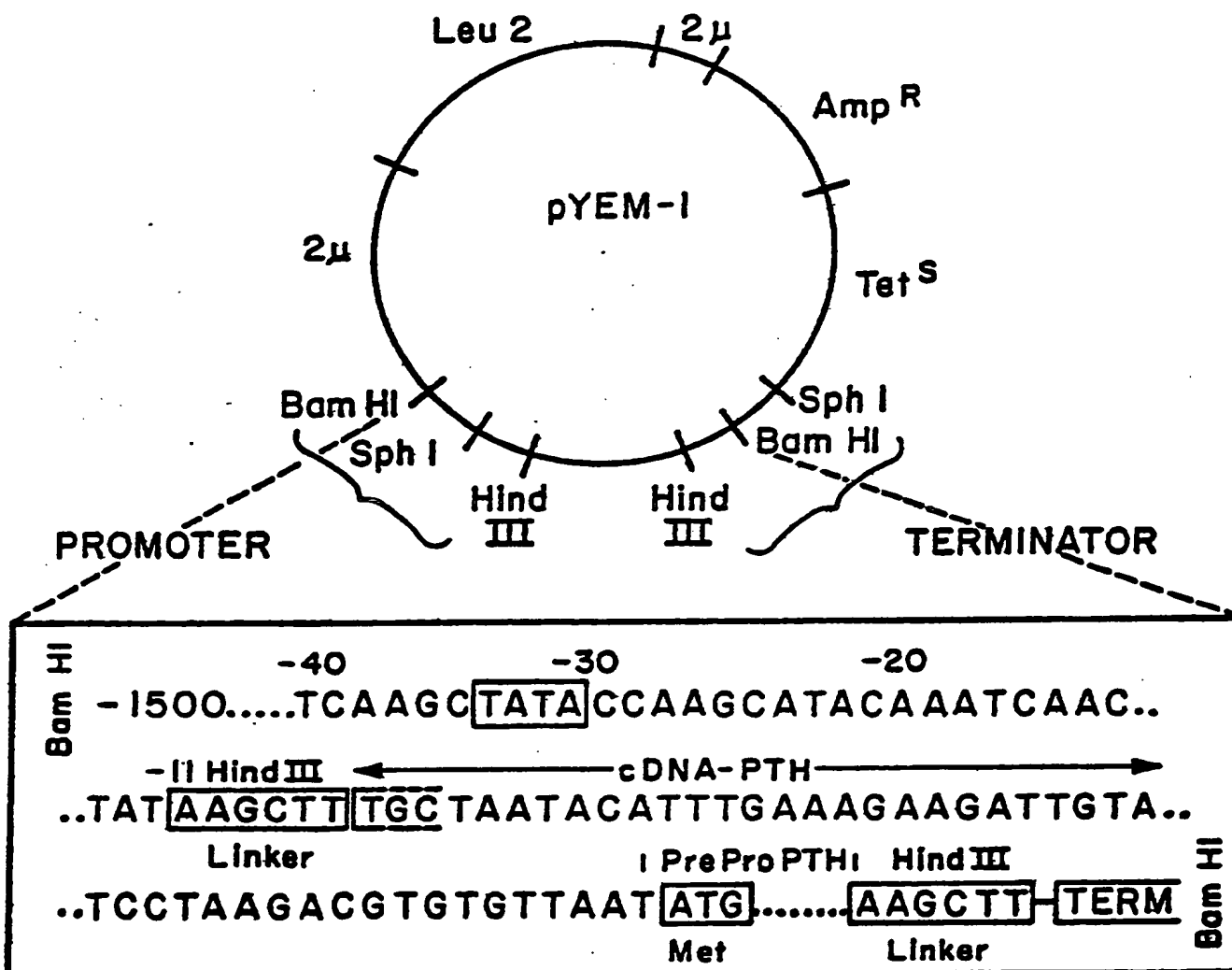
13. The method of claim 12 wherein the yeast is *Saccharomyces cerevisiae*.

14. A recombinant DNA plasmid transfer vector useful for transforming yeast comprising a DNA sequence comprising the preproparathyroid gene cDNA sequence.

15. The plasmid pYEM-1

16. Yeast transformed by a plasmid comprising the transfer vector of claim 14.

17. Yeast transformed by the plasmid of claim 15.

*Fig 1**Fig 3*

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PvuII cleavage site C/T

5' G GGG GGG GGG GGG GGG GGT TTA TCA GGC TTC TCA GGT TTA CTC AAC TTT GAG AAA GCA TCA GCT GCT AAT ACA TTT
 10 20 30 40 50 60 70

met met ser ala lys asp met val lys val met ile val met leu
 GAA AGA AGA TIG TAT OCT AAG ACG TGT GTT AAT ATG ATG TCT GCA AAA GAC ATG GTT AAG GTA ATG AAT GTC ATG CTT
 80 90 100 110 120 130 140 150

ala ile cys phe leu ala arg ser asp gly lys ser val lys lys arg ala val ser glu ile gln phe met his asn leu
 GGC ATC TGT TTT CTT GCA AGA TCA GAT GGG AAG TCT GGT AAG AGA GCT GTC AGT GAA ATA CAG TTT ATG CAT AAC CTG
 160 170 180 190 200 210 220 230

gly lys his leu ser ser met gly arg val glu trp leu arg lys lys leu gln asp val his asn phe val ala leu gly
 GGC AAA CAT CTG AGC TOC ATG GAA AGA AGA GTG GAA TGG CTG CCG AAA AAG CTA CAG GAT GTC CAC AAC TTT GTT GGC CTT GGA
 240 250 260 270 280 290 300 310

ala ser ile ala tyr arg asp gly ser ser gln arg pro arg lys lys glu asp asn val leu val glu ser his gln
 GCT TCT ATA GCT TAC AGA GAT GGT AGT TOC CAG AGA OCT CGA AAA AAG GAA GAC AAT GTC CTG GTT GAG AGC CAT CAG
 320 330 340 350 360 370 380 390

lys ser leu gly glu ala asp lys ala asp val leu ile lys ala lys pro gln stop
 AAA AGT CTT GGA GAA GCA GAC AAA GCT GAT GTC GAT GTA TTA ATT AAA GCT AAA CCC CAG TGA AAA CAG ATA TGA TCA GAT
 400 410 420 430 440 450 460 470

CAC TGT TCT AGA CAG CAT AGG GCA ACA ATA TTA CAT GCT GCT AAT GTC TTC ACC TTC TAT TAA GTC CCA GTA GTT CTA TGA
 480 490 500 510 520 530 540 550

Hinf cleavage site G/A

OCA ACC TTT ATT GCT AGC TGT GAT ACC TAC AAT TTT AAT TGA GTA TTT TGA TTC TAC TTT AAT CAT CTA AGA GCT CTT
 560 570 580 590 600 610 620 630

TTA ATA ATT CTA TTT CTA TTG ATT CCA AAT AAA TGA AGT TAA GTA TTA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
 640 650 660 670 680 690 700 710

AAA AAA AAA AAA AAA AAA CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC
 720 730 740 750 760 770 780 790 800

Fig 2

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US83/01361

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
INT. CL. C12P21/00, C12N15/00, 1/18, 1/00		
U.S. CL. 435/68, 172, 256, 317		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	435/68, 172, 256, 317	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
CHEMICAL ABSTRACTS FILES 308, 309, 310, 320 and 311 BIOSIS FILES 5, 55 and 255		
III. DOCUMENTS CONSIDERED TO BE RELEVANT **		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
Y	N, WILLIAMSON, GENETIC ENGINEERING 4 ACADEMIC PRESS PP108-125, 1983.	1-17
A	N, WALTON, RECOMBINANT DNA ELSEVIER SCIENTIFIC PUBLISHING CO., PP.185-197 and 213-227.	1-17
A	GB, A 2068969 A, PUBLISHED 19 AUGUST 1981.	1-17
<p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search *	Date of Mailing of this International Search Report *	
25 NOVEMBER 1983	06 DEC. 1983	
International Searching Authority *	Signature of Authorized Officer **	
ISA/US	ALVIN E. TANENHOLTZ	

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- ☐ 1 : [Olstad OK, Morrison NE, Jemtland R, Juppner H, Segre GV, Gautvik KM.](#) [Related Articles](#)
Differences in binding affinities of human PTH(1-84) do not alter biological potency: a comparison between chemically synthesized hormone, natural and mutant forms.
Peptides. 1994;15(7):1261-5.
PMID: 7854979; UI: 95158218

Related Resources

- ☐ 2 : [Olstad OK, Jemtland R, Loseth OP, Bringham FR, Gautvik KM.](#) [Related Articles](#)
Expression and characterization of a recombinant human parathyroid hormone partial agonist with antagonistic properties: Gly-hPTH(-1-->+84).
Peptides. 1995;16(6):1031-7.
PMID: 8532584; UI: 96087884
- ☐ 3 : [Pines M, Adams AE, Stueckle S, Bessalle R, Rashti-Behar V, Chorev M, Rosenblatt M, Suva LJ.](#) [Related Articles](#)
Generation and characterization of human kidney cell lines stably expressing recombinant human PTH/PTHrP receptor: lack of interaction with a C-terminal human PTH peptide.
Endocrinology. 1994 Oct;135(4):1713-6.
PMID: 7925136; UI: 95009736
- ☐ 4 : [Bringham FR, Juppner H, Guo J, Urena P, Potts JT Jr, Kronenberg HM, Abou-Samra AB, Segre GV.](#) [Related Articles](#)
Cloned, stably expressed parathyroid hormone (PTH)/PTH-related peptide receptors activate multiple messenger signals and biological responses in LLC-PK1 kidney cells.
Endocrinology. 1993 May;132(5):2090-8.
PMID: 8386606; UI: 93238632
- ☐ 5 : [Gardella TJ, Axelrod D, Rubin D, Keutmann HT, Potts JT Jr, Kronenberg HM, Nussbaum SR.](#) [Related Articles](#)
Mutational analysis of the receptor-activating region of human parathyroid hormone.
J Biol Chem. 1991 Jul 15;266(20):13141-6.
PMID: 1649179; UI: 91302339
- ☐ 6 : [Behar V, Pines M, Nakamoto C, Greenberg Z, Bisello A, Stueckle SM, Bessalle R, Usdin TB, Chorev M, Rosenblatt M, Suva LJ.](#) [Related Articles](#)
The human PTH2 receptor: binding and signal transduction properties of the

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L2: Entry 13 of 13

File: USPT

Apr 23, 1991

US-PAT-NO: 5010010

DOCUMENT-IDENTIFIER: US 5010010 A

TITLE: Production of human parathyroid hormone from microorganisms

DATE-ISSUED: April 23, 1991

INVENTOR-INFORMATION:

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Oven; Tordis B.	Oslo	N/A	N/A	NOX
Gabrielsen; Odd S.	Oslo	N/A	N/A	NOX

US-CL-CURRENT: 435/252.3; 435/252.33, 435/254.21, 435/320.1, 435/69.4, 435/69.9,
536/23.51

CLAIMS:

We claim:

1. A DNA sequence encoding *Saccharomyces* mating factor alpha 1 operably linked to human parathyroid hormone wherein said DNA sequence can stably transform a yeast cell to express and secrete an intact human parathyroid hormone.
2. The DNA sequence of claim 1 wherein said DNA sequence comprises the nucleotide sequence set forth in FIG. 11.
3. The yeast cell of claim 1 wherein said cell is in the genus *Saccharomyces*.
4. The yeast cell of claim 1 wherein said cell is of the species *Saccharomyces cerevisiae*.
5. The yeast cell of claim 1 wherein said cell is a budding yeast cell.
6. The human parathyroid hormone of claim 1 wherein said hormone has biological activity substantially equivalent to naturally occurring human parathyroid hormone.
7. A plasmid comprising the DNA sequence of claim 1.
8. A plasmid according to claim 7 wherein said nucleotide sequence comprises:
##STR2##
9. A plasmid according to claim 7 wherein the nucleotide sequence comprises:
##STR3##
10. A plasmid according to claim 7 wherein the nucleotide sequence comprises:
##STR4##
11. A plasmid according to claim 7 wherein the nucleotide sequence comprises:
##STR5##
12. A microorganism containing the plasmid of claim 8.
13. A microorganism according to claim 12 wherein said microorganism is *Escherichia coli*.
14. A microorganism containing the plasmid of claim 9.
15. A microorganism according to claim 14 wherein the microorganism is *Escherichia coli*.
16. A microorganism containing the plasmid of claim 10.
17. A microorganism according to claim 16 wherein the microorganism is *Escherichia coli*.
18. A microorganism containing the plasmid of claim 11.
19. A microorganism according to claim 18 wherein the microorganism is *Escherichia coli*.
20. A plasmid according to claim 7, wherein the nucleotide sequence comprises:
##STR6##

21. The plasmid of claim 7, wherein the nucleotide sequence comprises: ##STR7##
22. A microorganism containing the plasmid of claim 7.
23. A transformed yeast cell comprising a DNA sequence encoding *Saccharomyces* mating factor alpha 1 operably linked to human parathyroid hormone, said cell capable of expressing said DNA and secreting said expressed DNA into an extracellular environment, whereby said secreted, expressed DNA is intact human parathyroid hormone.
24. The transformed yeast cell of claim 23 wherein said DNA sequence comprises the nucleotide sequence of FIG. 11.
25. The transformed yeast cell of claim 23 wherein said yeast cell is of the genus *Saccharomyces*.
26. The transformed yeast cell of claim 23 wherein said yeast cell is of the species *Saccharomyces cerevisiae*.
27. The transformed yeast cell of claim 23 wherein said yeast cell is a budding yeast cell.
28. The transformed cell of claim 23 wherein said expressed human parathyroid hormone has a biological activity substantially equivalent to naturally occurring human parathyroid hormone.
29. A DNA sequence comprising a vector capable of stably transforming yeast wherein said DNA sequence encodes *Saccharomyces* mating factor alpha 1 operably linked to human parathyroid hormone wherein said vector can stably transform a yeast cell to express and secrete an intact human parathyroid hormone.
30. The transformed yeast cell of claim 29 wherein said yeast cell is a budding yeast.
31. The vector of claim 30 wherein said vector is an autonomous replicating plasmid.
32. The vector of claim 30 wherein said vector is an integrating plasmid.
33. The transformed yeast of claim 30 wherein said yeast is from the genus *Saccharomyces*.
34. The transformed yeast of claim 30 wherein said yeast is of the species *Saccharomyces cerevisiae*.
35. A plasmid, pSSHPTH-10, deposited in the American Type Culture Collection under ATCC No. 40267.
36. A transformed *E. coli* containing pSSHPTH-10, deposited in the American Type Culture Collection under ATCC No. 67223.
37. A transformed *S. cerevisiae* containing pSS.alpha.L.times.5-HPTH1, deposited in the American Type Culture Collection under ATCC No. 20821.
38. A plasmid, pSS.alpha.L.times.5-HPTH1, deposited in the American Type Culture Collection under ATCC No. 40266.

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L1: Entry 1 of 1

File: USPT

May 30, 1995

US-PAT-NO: 5420242

DOCUMENT-IDENTIFIER: US 5420242 A

TITLE: Production of human parathyroid hormone from microorganisms

DATE-ISSUED: May 30, 1995

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gautvik; Kaare M.	Oslo	N/A	N/A	NOX
Alestrom; Peter	Sollihøgda	N/A	N/A	NOX
Oyen; Tordis B.	Oslo	N/A	N/A	NOX

US-CL-CURRENT: 530/307; 536/23.51

CLAIMS:

We claim:

1. An isolated intact, functional human parathyroid hormone that is resistant to degradation by a KEX2 like proteolytic enzyme between the amino acids in positions 26 and 27 thereof.
2. The intact, functional human parathyroid hormone of claim 1 wherein Lys in position 26 is substituted with Gln.
3. An intact human parathyroid hormone operably linked to a modified Saccharomyces mating factor alpha 1 wherein a tetramer Glu-Ala-Glu-Ala, disposed immediately N-terminal to said human parathyroid hormone is omitted.
4. An intact human parathyroid hormone operably linked to the Lys Arg KEX2 cleavage site of the leader sequence of Saccharomyces mating factor alpha 1.
5. An intact human parathyroid hormone operably linked to the first 19 amino acids of Saccharomyces mating factor alpha 1.
6. A DNA sequence encoding Saccharomyces mating factor alpha 1 operably linked to a DNA sequence encoding human parathyroid hormone wherein said DNA sequence stably transforms a yeast cell to express and secrete an intact, functional human parathyroid hormone that is resistant to degradation by a KEX2 like proteolytic enzyme between the amino acids in positions 26 and 27 thereof.
7. The DNA sequence encoding Saccharomyces mating factor alpha 1 operably linked to a DNA sequence encoding human parathyroid hormone wherein said DNA sequence encoding human parathyroid hormone is modified such that the codon encoding Lys in position 26 thereof encodes Gln.
8. A DNA sequence encoding Saccharomyces mating factor alpha 1 operably linked to a DNA sequence encoding human parathyroid hormone wherein said DNA sequence encoding said mating factor alpha 1 is modified by deletion of the codons encoding the tetramer Glu-Ala-Glu-Ala disposed immediately N-terminal to said DNA sequence encoding human parathyroid hormone.
9. A DNA sequence encoding Saccharomyces mating factor alpha 1 and human parathyroid hormone wherein said DNA sequence encoding said human parathyroid hormone is operably linked to a DNA sequence encoding the Lys Arg KEX2 cleavage site of the leader sequence of Saccharomyces mating factor alpha 1.
10. A DNA sequence encoding the first 19 amino acids of Saccharomyces mating factor alpha 1 and human parathyroid hormone wherein the DNA sequence encoding human parathyroid hormone is operably linked to a portion of a DNA sequence encoding the first 19 amino acids of said Saccharomyces mating factor alpha 1 and wherein said DNA sequence encoding the first 19 amino acids of Saccharomyces mating factor alpha 1 and human parathyroid hormone which stably transforms a yeast cell to express and secrete an intact, functional human parathyroid hormone.

Chapter 9 Cloning in Yeast and other Microbial Eukaryotes

The analysis of eukaryotic DNA sequences has been facilitated by the ease with which DNA from eukaryotes can be cloned in prokaryotes using the vectors described in previous chapters. Such cloned sequences can be obtained easily in large amounts and can be altered *in vivo* by bacterial genetic techniques and *in vitro* by specific enzyme modifications. To determine the effects of these experimentally induced changes on the function and expression of eukaryotic genes, the rearranged sequences must be taken out of the bacteria in which they were cloned and reintroduced into a eukaryotic organism, preferably the one from which they were obtained. Despite the overall unity of biochemistry there are many functions common to eukaryotic cells which are absent from prokaryotes, e.g. localization of ATP-generating systems to mitochondria, association of DNA with histones, mitosis and meiosis, and obligate differentiation of cells. The genetic control of such functions must be assessed in a eukaryotic environment. In this chapter we will discuss the potential for cloning in the yeast *Saccharomyces cerevisiae* and in later chapters we will discuss the possibilities for cloning in animal and plant cells. It should be borne in mind that as well as using yeast as a host for cloned genes from other eukaryotes it also can be used as a host for analysing cloned yeast genes, i.e. surrogate yeast genetics.

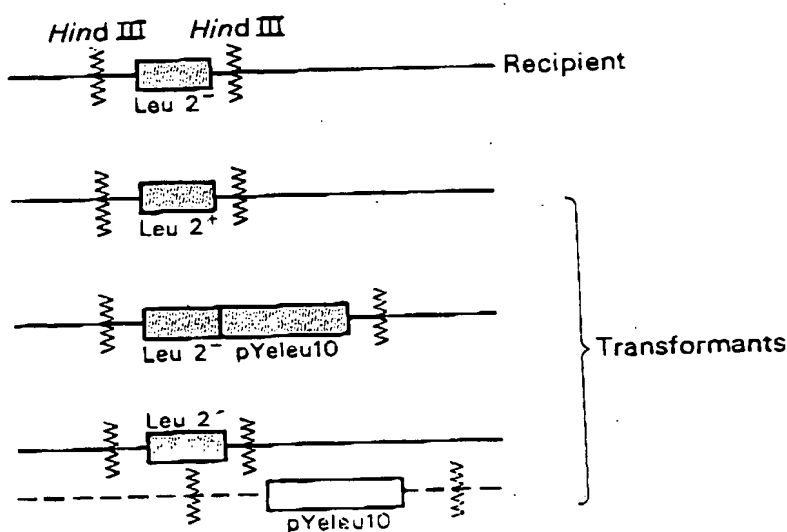
Transformation in yeast

Transformation of yeast was first achieved by Hinnen *et al.* (1978) who fused yeast spheroplasts (i.e. wall-less yeast cells) with polyethylene glycol in the presence of DNA and CaCl_2 and then allowed the spheroplasts to regenerate walls in a stabilizing medium containing 3% agar. The transforming DNA used was plasmid pYeLeu 10 which is a hybrid composed of the *E. coli* plasmid Col E1 and a segment of yeast DNA containing the Leu 2⁺ gene. Spheroplasts from a stable Leu 2⁻

auxotroph were transformed to prototrophy by this DNA at a frequency of 1×10^{-7} . Untreated spheroplasts reverted with a frequency of $< 1 \times 10^{-10}$. When 42 Leu^+ transformants were checked by hybridization, 35 of them contained Col E1 DNA sequences. Genetic analysis of the remaining seven transformants indicated that there had been reciprocal recombination between the incoming $\text{Leu } 2^+$ and the recipient $\text{Leu } 2^-$ alleles.

Of the 35 transformants containing Col E1 DNA sequences, genetic analysis showed that in 30 of them the $\text{Leu } 2^+$ allele was closely linked to the original $\text{Leu } 2^-$ allele whereas in the remaining 5, the $\text{Leu } 2^+$ allele was located on another chromosome. These results can be confirmed by restriction endonuclease analysis since pYeLeu 10 contains no cleavage sites for *Hind* III. When DNA from the $\text{Leu } 2^-$ parent was digested with endonuclease *Hind* III and electrophoresed in agarose, multiple DNA fragments were observed but only one of these hybridized with DNA from pYeLeu 10. With the 30 transformants in which the $\text{Leu } 2^-$ and $\text{Leu } 2^+$ alleles were linked, only a single fragment of DNA hybridized to pYeLeu 10 but this had an increased size consistent with the insertion of a complete pYeLeu 10 molecule into the original fragment. This data is consistent with their being a tandem duplication of the *Leu 2* region of the chromosome (Fig. 9.1). With the remaining

CHROMOSOME STRUCTURE OF TRANSFORMANTS AND RECIPIENT



ELECTROPHORETIC SEPARATION OF *Hind* III GENERATED FRAGMENTS WHICH HYBRIDIZE WITH pYeLeu 10

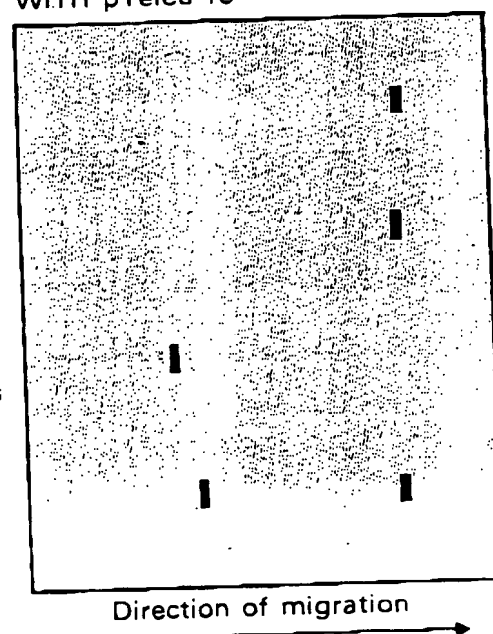


Fig. 9.1 Analysis of yeast transformants. See text for details.

five transformants, two DNA fragments which hybridized to pYeLeu 10 could be found on electrophoresis. One fragment corresponded to the fragment seen with DNA from the recipient cells, the other to the plasmid genome which had been inserted in another chromosome (Fig. 9.1). These results represented the first unambiguous demonstration that foreign DNA, in this case cloned Col E1 DNA, can integrate into the genome of a eukaryote. A plasmid such as pYeLeu 10 which can do this is known as a YIp-yeast integrating plasmid.

The development of yeast vectors:

1. Common principles

Since the first demonstration of transformation in yeast a number of different kinds of yeast vector have been constructed. All of them have features in common. First, all of them can replicate in *E. coli*, often at high copy number. This is important because for many experiments it is necessary to amplify the vector DNA in *E. coli* before transformation of the ultimate yeast recipient. Second, all employ markers, e.g. Leu 2⁺, His⁺, Ura 3⁺, Trp 1⁺, that can be selected readily in yeast and which often will complement the corresponding mutations in *E. coli* as well. In addition to these selectable markers most of the vectors also carry antibiotic resistance markers for use in *E. coli*. Finally, all of them contain unique target sites for a number of restriction endonucleases.

The development of yeast vectors:

2. Yeast episomal plasmids (YEp)

Many strains of *S. cerevisiae* contain a plasmid, 2 μ m long (6 kb), which has no known function. This plasmid replicates under nuclear control and there are approximately 50–100 copies per cell. It contains an inverted repeat sequence 600 base pairs long and is isolated as a mixture of two forms (Fig. 9.2) which differ from one another in the orientation of the two non-repeated segments of the molecule with respect to each other. The two forms are presumably due to recombination between the inverted repeat sequences (Hollenberg *et al.* 1976). Beggs (1978) constructed chimaeric plasmids containing this 2 μ m yeast plasmid, fragments of yeast nuclear DNA and the *E. coli* vector pMB9. These chimaeras were able to replicate in both *E. coli* and yeast, transformed yeast with high frequency, and some were able to complement auxotrophic mutations in yeast.

The chimaeric plasmids were constructed in two stages.

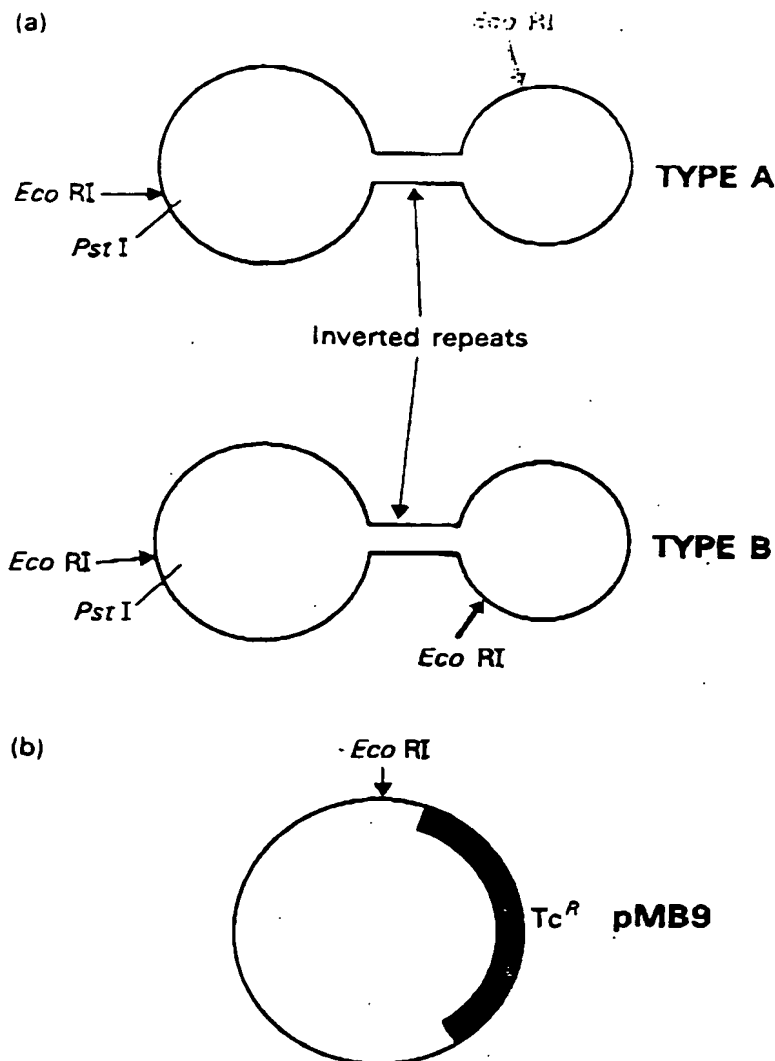


Fig. 9.2 Structures of (a) the yeast 2 μ m plasmid, and (b) pMB9. Only the cleavage sites for *Eco* RI and *Pst* I are shown.

First, plasmid pMB9 (Fig. 9.2) and the 2 μ m plasmid were joined by ligation of the DNA fragments produced by *Eco* RI endonuclease digestion. Tc^R clones were selected after transforming *E. coli* with the ligated DNAs and these were screened for a hybrid plasmid large enough to carry the complete yeast 2 μ m plasmid sequence. Yeast-pMB9 hybrid plasmids theoretically have eight possible configurations. These are determined by the orientation of the insertion into pMB9, which of the *Eco* RI sites on the yeast plasmid is used for insertion and whether the yeast sequence is in the A or B configuration. Five of the eight possible configurations, which can be distinguished by restriction mapping, were found among seven complete hybrid plasmids examined. The exact configuration of the yeast 2 μ m plasmid-pMB9 hybrids is probably not important.

For the second-stage Beggs (1978) sheared nuclear DNA isolated from *S. cerevisiae* and linked it by means of poly (dA-dT) tails to a mixture of pMB9-yeast 2 μ m plasmid hybrid plasmids which had been linearized by digestion with *Pst* I. Tc^R transformants were selected in *E. coli* and of the 21 000 obtained, two were found which complemented a Leu^- mutant. The plasmids from these clones, pJDB219 and pJDB248, transformed a *leuB* mutant of *E. coli* to Leu^+ or tetracycline resistance at the same frequency.

Both pJDB219 and pJDB248 transformed *Leu* 2 yeast mutants to Leu^+ with a frequency of 5×10^{-4} to 3×10^{-3} transformants per viable cell. This is several orders of magnitude greater than the frequency of transformation obtained by Hinnen *et al.* (1978). However, chromosomal integration of the transforming fragment was essential with the system of Hinnen *et al.* (1978). By contrast pJDB219 and pJDB248 could be recovered as plasmids from yeast cells and their inheritance in yeast was non-Mendelian.

Many other YE_p vectors based on the 2 μ m plasmid have been developed, e.g. Gerbaud *et al.* 1979, Storms *et al.* 1979, Struhl *et al.* 1979. They possess the same advantages as those of Beggs (1978), viz, they have a high copy number (25–100 copies $cell^{-1}$) in yeast and they transform yeast very well. Indeed, the transformation frequency can be increased 10- to 20-fold if single-stranded plasmids are used (Singh *et al.* 1982). The major problem associated with all YE_p vectors is that novel-rearranged recombinant plasmids are generated *in vivo* in *E. coli* and yeast.

The development of yeast vectors:

3. Yeast replicating plasmids (YRp)

Struhl *et al.* (1979) constructed a useful vector which consists of a 1.4 kb yeast DNA fragment containing the *trp-1* gene inserted into the *Eco* RI site of pBR322. This vector transformed *trp-1* yeast protoplasts to Trp^+ at high frequency and transforming sequences were always detected as ccc DNA molecules in yeast. No transformants were found in which the vector had integrated into the chromosomal DNA. Since pBR322 alone cannot replicate in yeast cells (Beggs 1978) a yeast chromosomal sequence must permit the vector to replicate autonomously and to express yeast structural genes in the absence of recombination with host chromosomal sequences. A similar vector based on pBR313 was developed by Kingsman *et al.* (1979). In both cases the yeast gene was linked to a centromere and initially it was thought that this was important. Since then it has been shown that a centromere is not essential; rather, the

vector carries an autonomously replicating sequence (*ars*) derived from the chromosome.

Although plasmids containing an *ars* transform yeast very efficiently the resulting transformants are exceedingly unstable, segregating at rates well above 1% per generation of growth in non-selective medium. Occasional stable transformants are found and these appear to be cases in which the entire YRp has integrated into a homologous region in a manner identical to that of YIps (Stinchcomb *et al.* 1979, Nasmyth & Reed 1980). The copy number of YRp vectors is much lower than that of YE_p vectors.

The development of yeast vectors:

4. Yeast centromere plasmids (YC_p)

Using a YRp vector Clarke and Carbon (1980) isolated a number of hybrid plasmids containing DNA segments from around the centromere-linked *leu2*, *cdc10* and *pgk* loci on chromosome III of yeast. As expected for plasmids carrying an *ars* most of the recombinants were unstable in yeast. However, one of them was maintained stably through mitosis and meiosis. The stability segment was confined to a 1.6 kb region lying between the *leu2* and *cdc10* loci and its presence on plasmids carrying either of 2 *ars* tested resulted in those plasmids behaving like minichromosomes (Clarke & Carbon 1980, Hsiao & Carbon 1981). Genetic markers on the minichromosomes acted as linked markers segregating in the first meiotic division as centromere-linked genes and were unlinked to genes on other chromosomes. Stinchcomb *et al.* (1982) and Fitzgerald-Hayes *et al.* (1982) have isolated the centromeres from chromosomes IV and XI of yeast and found that they confer on plasmids similar properties to the centromere of chromosome III. The isolation of plasmids bearing centromeres functional in yeast is a particularly exciting development for it should permit a detailed molecular analysis of the events occurring at mitosis and meiosis. Already a detailed analysis has been made of the molecular architecture of the centromere (Bloom & Carbon 1982).

Surrogate genetics of yeast

For many biologists the primary purpose of cloning is to understand what particular genes do *in vivo*. Thus most of the applications of yeast vectors have been in the surrogate genetics of yeast. One advantage of cloned genes is that they can be

analysed easily, particularly with the advent of DNA sequencing methods. Thus nucleotide sequencing analysis can reveal many of the elements which control expression of a gene as well as identifying the sequence of the gene product. In the case of the yeast actin gene (Gallwitz & Sures 1980, Ng & Abelson 1980) and some yeast tRNA genes (Abelson 1979, Olson 1981) this kind of analysis revealed the presence within these genes of non-coding sequences which are copied into primary transcripts. These *introns* subsequently are eliminated by a process known as *splicing* and this is discussed in more detail on page 200. Nucleotide sequence analysis also can reveal the direction of transcription of a gene although this can be determined *in vivo* by other methods; for example, if the yeast gene is expressed in *E. coli* using bacterial transcription signals, the direction of reading can be deduced by observing the orientation of a cloned fragment required to permit expression. Finally, if a single transcribed yeast gene is present on a vector the chimaera can be used as a probe for quantitative solution hybridization analysis of transcription of the gene.

The availability of different kinds of vectors with different properties (see Table 9.1) enables yeast geneticists to perform manipulations in yeast like those long available to *E. coli* geneticists with their sex factors and transducing phages. Thus cloned genes can be used in conventional genetic analysis by means of recombination using YIp vectors, or complementation using YE_p, YR_p or YC_p vectors. These latter vectors also permit the formation of partial diploids and partial polyploids. Transposition of genes can be done using integrative transformation and the new position of the gene determined by conventional Mendelian analysis. Deletions, point mutations and frame shift mutations can be introduced *in vitro* into cloned genes and the altered genes returned to yeast and used to replace the wild-type allele. Excellent reviews of these techniques have been presented by Botstein and Davis (1982), Hicks *et al.* (1982) and Struhl (1983).

Targeted selection of recombinant clones

In an analogous fashion to that used for prokaryotic genes, many yeast genes have been identified by transforming a mutant yeast strain with a recombinant library followed by screening or selecting for clones that complement the mutation of interest. For this approach to work it is essential to have a mutation in the gene of interest and a genetic marker that

Table 9.1 Properties of the different yeast vectors.

Vector	Transformation frequency	Loss in non-selective medium	Disadvantages	Advantages
YIp	1-10 transformants per μg DNA	Much less than 1% per generation	<p>(1) Low transformation frequency</p> <p>(2) Can only be recovered from yeast by cutting chromosomal DNA with restriction endonuclease which does not cleave original vector containing cloned gene</p>	<p>(1) Of all vectors this kind give most stable maintenance of cloned genes</p> <p>(2) An integrated YIp plasmid behaves as an ordinary genetic marker, e.g. a diploid heterozygous for an integrated plasmid segregates the plasmid in a Mendelian fashion.</p> <p>(3) Most useful for surrogate genetics of yeast, e.g. can be used to introduce deletions, inversions and transpositions (see Botstein & Davis 1982)</p>
YE _p	10^3 - 10^5 transformants per μg DNA	1% per generation	<p>(1) Novel recombinants generated <i>in vivo</i> by recombination with endogenous 2 μm plasmid</p>	<p>(1) Readily recovered from yeast</p> <p>(2) High copy number</p> <p>(3) High transformation frequency</p> <p>(4) Very useful for complementation studies</p>

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YRp

10^2-10^3
transformants
per μg DNA

Much greater
than 1% per
generation but
can get
chromosomal
integration

(1) Instability of transformants

- (1) Readily recovered from yeast
- (2) High copy number. Note that the copy number is usually less than that of YEp vectors but this may be useful if cloning gene whose product is deleterious to the cell if produced in excess
- (3) High transformation frequency
- (4) Very useful for complementation studies
- (5) Can integrate into the chromosome

YCp

10^2-10^3
transformants
per μg DNA

Less than 1%
per generation

- (1) Low copy number makes recovery from yeast more difficult than that of YEp or YRp vectors

- (1) Low copy number is useful if product of cloned gene is deleterious to cell
- (2) High transformation frequency
- (3) Very useful for complementation studies
- (4) At meiosis generally shows Mendelian segregation

can be used to select the desired transformants. However, despite the fact that *S. cerevisiae* has been intensively studied genetically only a small fraction of the genes encoding enzymes has been identified by mutation. Furthermore, there are several examples of two or more yeast genes encoding either the same product or functionally homologous products or multiple coding sequences for the same product, e.g. alcohol dehydrogenase. Such duplication makes it difficult to obtain suitable mutants. Rine *et al.* (1983) have presented an alternative approach to complementation for isolating genes. Their method is based on the observation that it is possible to overcome the effects of an inhibitor by increasing the dosage of the gene encoding the target protein. In practice, the concentration of the inhibitor which is just sufficient to prevent growth of wild-type yeast cells is determined and the transformants are selected which are resistant to this concentration of inhibitor. In this way Rine *et al.* (1983) were able to clone genes conferring resistance to tunicamycin (an inhibitor of UDP-N-acetyl glucosamine-1-P transferase), compactin (an inhibitor of 3-hydroxy-3-methyl-glutamyl-CoA reductase), and ethionine (an inhibitor of S-adenosyl methionine synthetase).

Linear vectors for cloning yeast telomeres

All three autonomous plasmid vectors are maintained in yeast as circular DNA molecules—even the YCp vectors which possess yeast centromeres. Thus none of these vectors can model the eukaryotic chromosome which is a linear structure. A model system is of particular interest as the biochemical mechanism by which the ends of linear DNA molecules are replicated is unknown (see Watson 1972, for detailed discussion). Szostak and Blackburn (1982) have developed such a model system by using a linear vector to clone yeast telomeres.

The linear yeast vector was constructed from two components. The first was a YRp, pSZ213 (Fig. 9.3) which has no *Bam* HI sites and a single *Bgl* II site. The second component was fragments of an unusual, linear, rRNA-encoding plasmid found in the protozoan *Tetrahymena*. This plasmid DNA was cleaved with *Bam* HI and the end fragments were ligated to *Bgl* II-cut pSZ213. Since *Bam* HI-cut and *Bgl* II-cut ends of DNA are complementary they can be joined by ligase but the product of ligation is not a substrate for either enzyme. Consequently both *Bam* HI and *Bgl* II endonucleases were present in the ligation mixture to cut circularized or dimerized vector molecules and

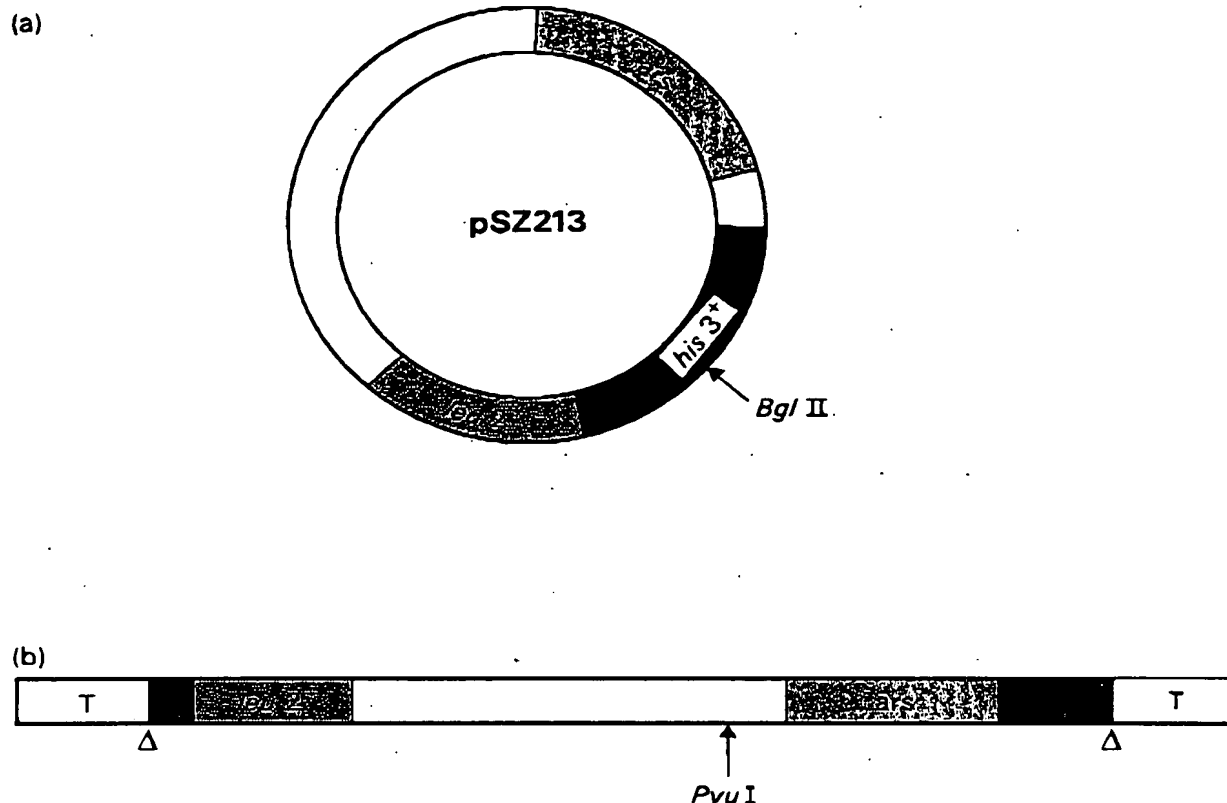


Fig. 9.3 (a) Simplified map of plasmid pSZ213 used for cloning yeast telomeres. The plasmid has no *Bam* HI sites and insertion of telomeric DNA at the unique *Bgl* II site inactivates the *his3*⁺ gene. (b) Structure of a linear plasmid constructed from pSZ213 showing the asymmetric location of the unique *Pvu* I site. The open triangles indicate the location of *Bam*/*Bgl* joints created when the telomeric DNA (indicated with a T) was added to *Bgl* II-digested pSZ213.

dimerized end fragments. In this way the desired linear vector carrying *Tetrahymena* telomeres accumulated in the reaction mixture and it was purified by agarose gel electrophoresis.

The linear plasmid containing the *Tetrahymena* telomeres retains the *Leu2*⁺ marker and this was used for selection of transformants in yeast. Since YRp vectors are capable of integration, those transformants in which the linear plasmid was replicating autonomously were detected by their mitotic instability. Restriction mapping showed that the plasmid in the unstable transformants is a linear molecule identical in structure to the linear molecule constructed *in vitro* and used in the transformation. Furthermore, the ends of *Tetrahymena* rDNA have three unusual structural features: a variable number of short, 5'-CCCCAA-3' repeat units, specific single-strand interruptions within the repeated sequences and a cross-linked

terminus. All three structural features were maintained when the *Tetrahymena* sequences were cloned in yeast.

The linear plasmid carrying the *Tetrahymena* telomeres has a single, asymmetrically placed target site for endonuclease *Pvu* I. This enzyme cuts yeast DNA into approximately 2000 fragments and since yeast has 17 chromosomes 34 of these fragments should contain telomeres. After ligating *Pvu* I-digested chromosomal and plasmid DNA unstable *Leu*⁺ transformants were selected. Many of the plasmids generated in this way would have multiple yeast *Pvu* I fragments ligated onto the vector followed by either a yeast end or an end derived from the vector. These plasmids would be as large as or larger than the original vector. In contrast, the ligation of a single *Pvu* I telomere fragment from yeast, smaller than the *Tetrahymena*-containing fragment being replaced, would yield a linear plasmid smaller than at the start. After size screening of the plasmids from the transformants three plasmids carrying a yeast telomere at one end were identified. Analysis of these plasmids shows that yeast telomeres have at least some of the structural features of *Tetrahymena* telomeres. Furthermore, their use as hybridization probes suggests that all yeast telomeres are structurally similar.

Stability of yeast cloning vectors

As noted earlier, YRp vectors are not stably maintained by yeast cells and, in the absence of selection, are quickly lost from the population. By contrast, the segregational stability of YCp vectors which carry a yeast centromere is considerably greater than that of YRp vectors. Murray and Szostak (1983a) have found that YRp vectors have a strong bias to segregate to the mother cell at mitosis. This segregation bias explains how the fraction of plasmid-bearing cells can be small despite the high average copy number of YRp vectors. The presence of a centromere eliminates segregation bias thus accounting for the increased stability of YCp vectors relative to YRp vectors. YEpl vectors are stably maintained in yeast cells provided the strain contains endogenous intact 2 μ m circles. In the absence of endogenous 2 μ m circles YEpl vectors show maternal segregation bias.

Despite the fact that YCp vectors are relatively stable, they are still 1000 times less stable than *bona fide* yeast chromosomes. However, YCp vectors and YRp vectors are circular molecules whereas chromosomes are linear molecules. A linear plasmid vector carrying a centromere would be much more represen-

tative of a yeast chromosome. Dani and Zakian (1983) constructed linear yeast plasmids but found that stability was reduced relative to the circular plasmid. However, Murray and Szostak (1983b) found that stability of linear yeast plasmids was related to size. Thus artificial yeast chromosomes which were 55 kb long and contained cloned genes, *ars*, centromeres and telomeres had many of the properties of natural yeast chromosomes. When the artificial chromosomes were less than 20 kb in size, centromere function was impaired.

Whereas the stability of linear YCp vectors is dependent on size this is not true of YRp vectors. Murray and Szostak (1983a) constructed a linear YRp vector and found that it did not exhibit maternal segregational bias. The model used by them to explain these results is as follows. During DNA replication there is an association between *ars* elements and fixed nuclear sites. Replicated molecules remain attached to this site which is destined to segregate to the mother cell. The replicated plasmids will exist initially as catenated dimers which subsequently are resolved. If the dimers are attached to the putative segregation site by only one of their constituent monomers, their resolution by topoisomerase activity would release one monomer from each dimer and this monomer would be free to segregate at random to either the mother or the daughter cell. With linear plasmids, replication will produce two linear molecules which are not interconnected. Thus prior to mitosis there will be at least one freely segregating molecule and this explains the increased stability of linear YRp vectors.

Expression of cloned genes in yeast

As might be expected, most cloned yeast genes are expressed when reintroduced into yeast. More surprising, some bacterial genes are also expressed in yeast (Cohen *et al.* 1980, Jimenez & Davies 1980) and in one instance expression was dependent on a bacterial promoter (Breunig *et al.* 1982). Since Struhl and Davies (1980) showed that a yeast promoter is functional in *E. coli* it might be thought that transcription signals such as promoters can be active in prokaryotes and eukaryotes. However, this clearly is not the case for a number of workers failed to get expression of foreign genes in yeast. Thus Rose *et al.* (1981) obtained expression of β -galactosidase in *E. coli* when it was under the control of either the *E. coli* Tc^R promoter or the yeast *ura* 3 promoter but achieved expression in yeast only with the latter promoter. When Beggs *et al.* (1980) introduced the rabbit β -globin gene into yeast, β -globin-specific transcripts

were obtained but transcription started at a position downstream from the usual initiation site. Finally, even though a *Drosophila* gene corresponding to the yeast *ade8* locus has been identified by complementation, *Drosophila* genes complementing mutants at other yeast loci have not been obtained (Henikoff *et al.* 1981).

Use of yeast promoters

Because of difficulties in obtaining heterologous gene expression in yeast a number of groups have turned to the use of yeast promoters and translation initiation signals. Thus expression of the *E. coli* β -galactosidase gene was obtained by fusing it to the N-terminus of the *ura3*, *cyc1* and *arg3* genes (Guarente & Ptashne 1981), Rose *et al.* 1981, Crabeel *et al.* 1983). Expression in yeast of an interferon-alpha gene was obtained by fusing it to either the *pgk* or *trp1* genes (Tuite *et al.* 1982, Dobson *et al.* 1983). In many instances, expression of a mature protein rather than a fusion protein is required. To achieve this Hitzeman *et al.* (1981) started with a plasmid carrying the promoter and part of the coding sequence of the *adh1* gene (Fig. 9.4). This plasmid was cut with endonuclease *Xho* I for which there is a unique cleavage site downstream from the initiating ATG codon. The linearized vector was digested with nuclease *Bal* 31 to remove 30–70bp of DNA from each end of the molecule. The DNA then was incubated with the Klenow fragment of DNA polymerase and deoxynucleoside triphosphates to fill in the ends and synthetic *Eco* RI linkers added. After cleavage with endonucleases *Eco* RI and *Bam* HI the assorted fragments containing variously deleted *adh1* promoter sequences were isolated by preparative electrophoresis. In this way six different promoter fragments were isolated, joined to an interferon-alpha gene and used to direct the synthesis of mature interferon in yeast. In a similar fashion synthesis of mature hepatitis B virus surface antigen was achieved from the *adh1* promoter (Valenzuela *et al.* 1982) and mature interferon-gamma from the *pgk* promoter (Derynck *et al.* 1983).

Factors affecting expression

In all three instances where a mature foreign protein was synthesized in yeast the levels of expression were considerably less than that of yeast alcohol dehydrogenase I (*adh1*) or 3-phosphoglycerate kinase (*pgk*). There are a number of explanations; for example, Derynck *et al.* (1983) found that transcription was greatly reduced. Since the distance between

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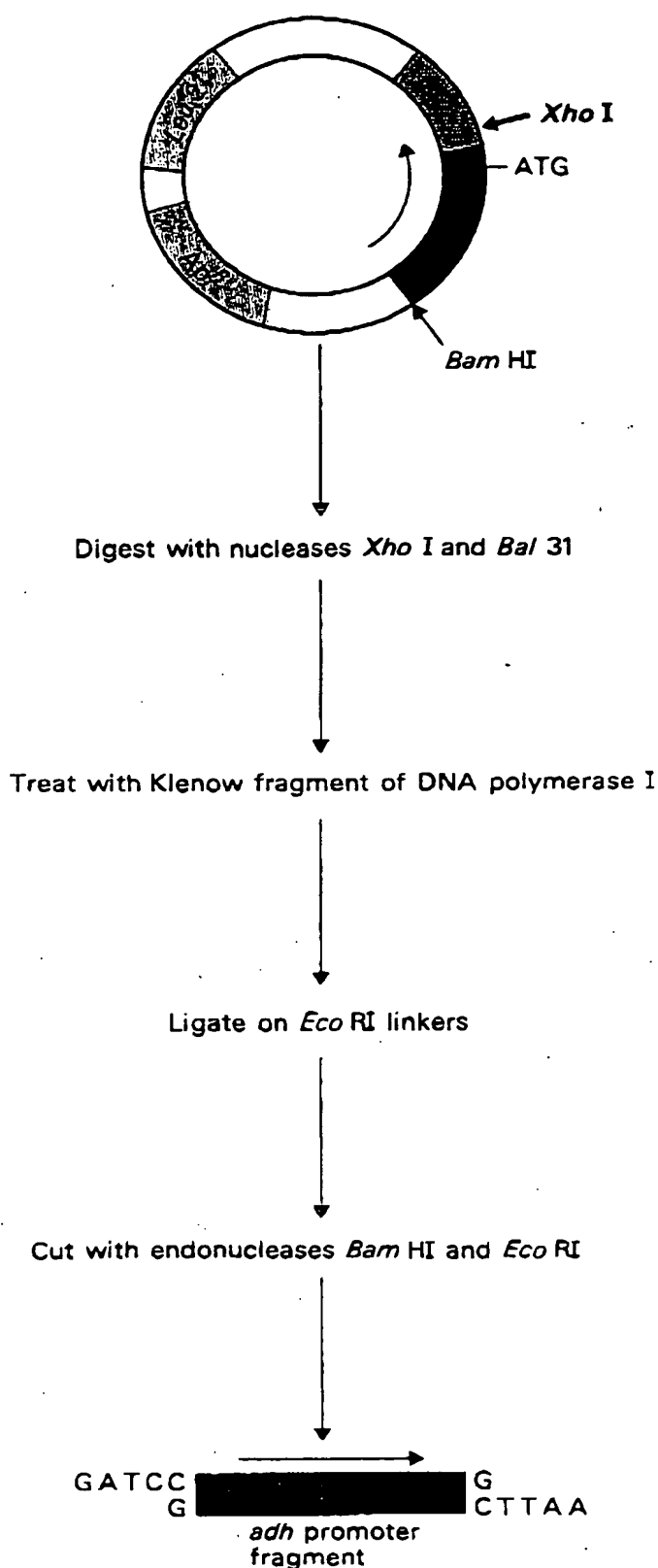


Fig. 9.4 The procedure used to generate *adh* promoter fragments. The *adh* promoter region in the starting plasmid is indicated by the black area. The grey area represents another gene fused to the N-terminus of the *adh* gene. The distance between the unique *Xho*I site and the initiating ATG codon is 17bp. The arrow inside the plasmid circle (top) and above the *adh* promoter fragment (bottom) indicates the direction of transcription.

the promoter and initiating ATG codon is less than usual and the base sequences in the vicinity of the ATG codon are different, translation initiation might be reduced (c.f. *E. coli*, p. 141). Also, translation might be inefficient due to strong differences in codon usage between human and yeast genes, particularly since there is an extreme codon bias in highly expressed yeast genes (Bennetzen & Hall 1982). Stepien *et al.* (1983) were unable to obtain detectable expression of proinsulin from a synthetic gene under the control of the yeast *adh* promoter, in marked contrast to the results obtained with interferon and hepatitis B surface antigen. Since proinsulin-specific mRNA was abundant, poor translation due to the absence of preferred codons could be an explanation. A more likely explanation is proteolysis of the proinsulin because detectable synthesis of proinsulin was obtained when the proinsulin gene was fused in phase with the yeast galactokinase gene. This result is analogous to that obtained in *E. coli* with β -galactosidase-somatostatin gene fusions (see p. 64).

In an attempt to identify those structural features which control expression of yeast genes, Dobson *et al.* (1982) compared the nucleotide sequence of the 5' flanking regions of 17 yeast genes. One sequence thought to be important for transcription initiation in eukaryotes was found in most of the yeast genes. This is the TATA box which is an AT-rich region with the canonical sequence TATAT/AAT/A usually located 25–32bp upstream from the transcription initiation site. Functionally it is equivalent to the Pribnow box of prokaryotes (see p. 139). Other sequences thought to be important for transcription initiation in eukaryotes were missing. With regard to translation initiation all the yeast genes had an adenine residue at position -3 and all except one had a pyrimidine, usually thymine, at position +6. The poor expression of mature hepatitis antigen and interferons alpha and gamma could be explained by poor translation initiation since in each case one of these residues was not conserved.

Ideally, cloned genes should be placed downstream from a controllable promoter and this is what Kramer *et al.* (1984) have done. They constructed a suitable vector using the promoter/regulator region of the repressible acid phosphatase gene of yeast. An interferon gene was inserted into this vector. Yeast cells transformed with the resulting plasmid chimaera produced significant amounts of interferon only when grown in medium lacking inorganic phosphate. Furthermore, mutants in two acid phosphatase regulator genes (coding for a defective repressor and a temperature-sensitive positive regulator) were used to develop a yeast strain that grew well at 35°C but

produced interferon only at 23°C, independent of the phosphate concentration.

Stetler and Thorner (1984) have developed a method for identifying yeast genes whose transcription is differentially regulated. The method is based on incorporation of the analogue 4-thiouridine into nascent RNAs which are purified by affinity chromatography on phenylmercury agarose. The purified RNAs are used to prepare cDNA copies for screening of genomic DNA libraries by hybridization. Using this procedure several cloned yeast DNA segments were found whose transcription *in vivo* was modulated by exposure to mating hormone. These hormone-responsive genes fell into 3 major classes: first, genes expressed in vegetatively growing cells that were no longer transcribed after administration of mating factor; second, genes whose expression was increased 10 to 20-fold after exposure to the mating hormone; finally, genes which were expressed only after mating factor treatment.

It must be realized that control of gene expression in yeast is much more complicated than is apparent from the above discussion. In-vitro mutagenesis of cloned genes followed by their reintroduction into yeast has resulted in the identification of *cis*-acting elements that modulate transcription despite the fact that they are located hundreds of nucleotides upstream of the site of transcription initiation. For a review of this topic the reader should consult Guarente (1984).

Secretion of proteins by yeast

Secretion of proteins synthesized in yeast can be achieved by the addition of a signal sequence. Hitzeman *et al.* (1983) constructed a series of plasmids in which either mature interferon or pre-interferon genes were placed downstream from a *pgk* promoter such that native, as opposed to fused, proteins were produced. Yeast cells carrying such plasmids synthesized interferon but only in those encoding pre-interferon was any interferon activity found in the medium. Sequencing of interferons purified from the growth medium showed that they had the same amino termini as the natural mature interferons. This result shows that yeast cells can recognize and correctly process a signal sequence from a higher eukaryote.

Targeting of proteins to the nucleus

The yeast nucleus contains a discrete set of proteins which are synthesized in the cytoplasm. In order to elucidate the mech-

anism governing nuclear protein localization Hall *et al.* (1984) constructed a set of hybrid genes by fusing the yeast MAT alpha 2 gene, encoding a presumptive nuclear protein, and the *E. coli* *lacZ* gene. A segment of the MAT alpha 2 gene product which was 13 amino acids long was sufficient to localize beta-galactosidase activity in the nucleus. The nuclear location of the beta-galactosidase was confirmed by immunofluorescence.

Excision of introns by yeast

Many eukaryotic genes contain non-coding regions called introns (see p. 189). Introns have two common structure features: their sequences begin with the dinucleotide 5'-GT-3' and end with the dinucleotide 5'-AG-3'. Besides these invariant nucleotides there is limited structural similarity at and around the intron-exon junction and consensus sequences have been derived from comparison of more than 100 junctions. Because of the similarity of these junction sequences in widely different species, e.g. yeast and man, it was thought that the mechanism of RNA processing to remove introns might be universal. Support for this idea came from the observation that monkey cells can splice out introns from mouse and rat genes, and mouse cells correctly splice transcripts of rabbit and chicken genes. This raises the question whether yeast cells can remove introns from genes of higher eukaryotes. If so this would be of great practical value, for the presence of introns prevents shotgun cloning of functional eukaryotic genes in *E. coli*.

To test the ability of yeast cells to excise introns from foreign genes Beggs *et al.* (1980) transformed *S. cerevisiae* with a hybrid plasmid containing a cloned rabbit chromosomal DNA segment including a complete β -globin gene with two intervening sequences and extended flanking regions. Yeast cells transformed with this chimera produced β -globin specific mRNA. However, these globin transcripts were about 20-40 nucleotides shorter at the 5' end than normal globin mRNA, contained one intron and extended only as far as the first half of the second intron. This result could be taken to indicate that the splicing mechanisms in yeast and rabbit differ but it could be argued that a complete transcript is a prerequisite for RNA splicing and that the prematurely terminated globin RNA was not a substrate for the yeast splicing enzyme(s). Consequently, Langford *et al.* (1983) inserted into the intron-containing yeast actin gene an intron-containing fragment from either *Acanthamoeba* or duck. In both instances yeast cells removed the

natural yeast intron but not the foreign intron from the chimaeric transcript.

Why are introns in foreign genes not removed by yeast cells? The sequence of the coding regions surrounding the splice site cannot be important, for Teem and Rosbash (1983) have inserted a yeast intron into the *E. coli* β -galactosidase gene and found that it is removed correctly in yeast cells. The most likely explanation is that sequences within the intron are required for correct splicing in yeast. In this context Langford and Gallwitz (1983) have found an octanucleotide (5'-TACT AACA-3') which occurs 20 to 55 nucleotides upstream from the 3' splice site in all split protein-coding genes of *S. cerevisiae* analysed and which is absent from most introns of higher eukaryotes. Single A \rightarrow C transversions in the fourth or eighth position of this sequence prevent splicing from occurring (Langford *et al.* 1984).

CLONING IN OTHER MICROBIAL EUKARYOTES

Compared with yeast there has been relatively little development of gene cloning systems in filamentous fungi. In an analogous experiment to that of Hinnen *et al.* (1978), Case *et al.* (1979) were able to transform *Neurospora crassa* with a cloned *qa-2⁺* gene, encoding catabolic dehydroquinase. As with yeast, three types of transformation event were distinguished: replacement of the *qa-2⁻* gene with the *qa-2⁺* gene, linked insertion of the *qa-2⁺* gene, and unlinked insertion. Shuttle vectors have been developed by Stohl and Lambowitz (1983) and Hughes *et al.* (1983) for the transfer of genes between *E. coli* and *N. crassa*. Southern blots show that these shuttle vectors are present in nuclear and cytosolic fractions of *Neurospora* transformants. As might be expected the frequencies of transformation of *Neurospora* with these shuttle vectors is 5- to 10-fold higher than for plasmids that transform mainly by integration.

By a modification of the procedure of Hinnen *et al.* (1978) Ballance *et al.* (1983) were able to transform *Aspergillus nidulans* with a pBR322-borne *pyr-4* gene of *N. crassa*. The available evidence suggests that the *pyr-4* gene and at least some of the pBR322 had integrated into the chromosome of the *A. nidulans* transformants. In a more definitive study Yelton *et al.* (1984) have shown that a cloned *A. nidulans trpC* gene becomes integrated into the *A. nidulans* chromosome following transformation.

Transformation of *Podospora* has been achieved with a hybrid plasmid consisting of *E. coli* plasmid pBR325 and defective mtDNA (Stahl *et al.* 1982). This hybrid plasmid replicated auton-

omously in *Podospora* where it expressed the *Podospora* senescence trait which is the successful competition of defective mtDNA with wild-type mtDNA.

Plasmids that replicate autonomously in the unicellular green alga *Chlamydomonas reinhardtii* have been constructed by inserting random DNA fragments from this alga into a plasmid containing the yeast *arg 4* locus. These plasmids transformed an *arg* auxotroph of *C. reinhardtii* to prototrophy (Rochaix *et al.* 1984). The presence of free plasmids in the transformants was demonstrated by hybridisation with a specific plasmid probe and by recovering the plasmids in *E. coli*. Analysis of the plasmids showed that they contained chloroplast DNA sequences functionally equivalent to the *arg* sequence of yeast.

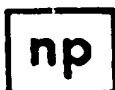
YEAST MOLECULAR BIOLOGY— RECOMBINANT DNA

Recent Advances

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Recombinant DNA

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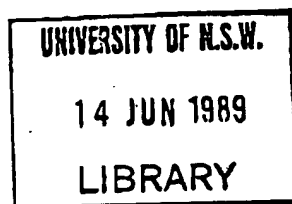
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COMPARISON OF REVERSED-PHASE AND CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SEPARATING CLOSELY RELATED PEPTIDES: SEPARATION OF ASP⁷⁶-HUMAN PARATHYROID HORMONE (1-84) FROM ASN⁷⁶-HUMAN PARATHYROID HORMONE (1-84)

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SUMMARY

Cation-exchange high-performance liquid chromatography (CE-HPLC) was compared with ordinary reversed-phase high-performance liquid chromatography (RP-HPLC) for separating closely related peptides. Some synthetic samples of bradykinin and angiotensins, which were homogeneous according to RP-HPLC, were found to be inhomogeneous when analyzed by CE-HPLC. On the other hand, diastereomeric peptides could be separated much more efficiently by RP-HPLC than by CE-HPLC. These results indicated that the purity of synthetic peptides should be checked not only by RP-HPLC but also by ion-exchange HPLC. In the case of human parathyroid hormone (hPTH), baseline separation of Asp⁷⁶-hPTH from Asn⁷⁶-hPTH by RP-HPLC was not possible, but was by CE-HPLC. Using this method we confirmed that the Asn residue in hPTH at position 76 could not be converted into the Asp residue under the conditions used to isolate and purify it from human organs.

INTRODUCTION

In 1978, Keutmann *et al.*¹ found the amino acid sequence of human parathyroid hormone (hPTH) to be a linear peptide with 84 amino acid residues when they applied Edman degradation reactions to an isolated hormone. The structure of the same peptide was deduced by Hendy *et al.*² from sequence analysis of the cDNA; both structures were identical except for the residue at position 76, which was thought to be Asp from the Edman degradation analysis and Asn from the DNA analysis. When the new structure was reported, the previous one with Asp at position 76 was suspected to be an artifact which might have been formed by spontaneous deamidation of the Asn residue during isolation or purification of the natural peptide. To verify this, we synthesized both Asn⁷⁶-hPTH and Asp⁷⁶-hPTH by the solution procedure^{3,4}, and examined their separation by ordinary reversed-phase high-performance liquid chromatography (RP-HPLC). Under isocratic conditions, the hormones

were eluted very close together but with clearly different retention times. However, when injected into the same column as a mixture, the peptides were eluted together as a rather broad peak^{4,5}. In the present study we have tried to establish conditions for separating such closely related peptides by HPLC.

Recently, ion-exchange type columns have been introduced for HPLC and their usefulness in separating peptides and proteins has been reported^{6,7}. In order to elucidate the characteristic features of the new technique, we compared the resolving power of cation-exchange HPLC (CE-HPLC) with that of ordinary RP-HPLC by using some synthetic peptides as test samples. We also used the CE-HPLC technique to find the conditions for separation of the two hPTH analogues, and then applied them to determine whether or not Asp⁷⁶-hPTH is an artifact formed during isolation of hPTH from organs.

EXPERIMENTAL

Materials

Commercial samples of angiotensin I (AngI) and bradykinin (BK) were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), UCB Bioproducts (Bruxelles, Belgium) and Bachem (Bubendorf, Switzerland). Other peptides were synthesized by solution procedures in our laboratory using previously reported methods⁸. The reagents and solvents for chromatography were of HPLC-reagent grade. The water used was distilled in a Toyo Aquarius Model GS-20N still (Toyo Kagakusangyo, Tokyo, Japan).

CE-HPLC

CE-HPLC was carried out on a Shimadzu liquid chromatograph Model LC-4A (Kyoto, Japan) equipped with a Rheodyne 7125 syringe-loading sample injector, a Shimadzu variable-wavelength UV detector Model SPD-2AS, a Shimadzu column oven Model CTO-2AS and a Shimadzu data processor Chromatopac C-R2AX. The column (250 × 4.6 mm I.D.) was packed with a cation-exchange resin, TSK gel CM-2SW (Toyo Soda, Tokyo, Japan). Two solvent systems were used: A, 10% acetonitrile in 20 mM sodium phosphate buffer (pH 6.0); B, 10% acetonitrile in 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M sodium chloride. Elution was performed at 42°C at a flow-rate of 0.8 ml/min; other conditions are as stated in the Figs.

RP-HPLC

RP-HPLC was performed on a Hitachi liquid chromatograph Model 638-30 equipped with a multi-wavelength UV monitor Model 635M (Tokyo, Japan); the column (150 × 4.0 mm I.D.) was packed with Nucleosil 5 C₁₈ (Macherey-Nagel, Düren, F.R.G.). Chromatography was carried out at a flow-rate of 1.0 ml/min at ambient temperature unless stated otherwise.

Test of conversion of Asn⁷⁶-hPTH into Asp⁷⁶-hPTH

Asn⁷⁶-hPTH (15 µg) was dissolved in 75 µl each of water, 0.1 M ammonium acetate buffer (pH 2.5, 5.0 or 7.5), 0.1 M ammonium hydrogen carbonate buffer (pH 8.7) or 4% trichloroacetic acid (TCA). After the solution had been kept for 1 day or

5 days at room temperature, 35 μ l from each solution were lyophilized twice; the residue was dissolved in 20 mM sodium phosphate buffer (pH 6.0) and subjected to HPLC. With the TCA solution, the whole mixture was lyophilized after it had been kept for 1 day at room temperature, and the residue was subjected to HPLC after it had been dissolved in a 20 mM sodium phosphate buffer (pH 6.0).

RESULTS AND DISCUSSION

As reported previously⁹, various angiotensin II (AngII) analogues, such as β -Asp¹-, D-Asp¹-, D-Tyr⁴-, Val⁵-, Leu⁵-, des-Ile⁵-, D-His⁶- and D-Phe⁸-AngII, were clearly separated by RP-HPLC, except for β -Asp¹-AngII, which is always eluted together with Asp¹-AngII in our RP-HPLC system. The separation could be slightly improved by increasing the pH of the buffer system, but it was far from a baseline separation. CE-HPLC enabled a clear separation of the two AngII analogues as shown in Fig. 1. These results indicate that the purity of synthetic peptides containing Asp residue(s) should be checked not only by RP-HPLC but also by CE-HPLC. Particularly important is the detection of β -Asp-containing peptide since some Asp residue(s) in peptides have a great tendency to be converted into β -Asp residue(s) during various steps of their synthesis. We applied these techniques to test the homogeneity of commercially available samples of AngI. As expected in RP-HPLC, all samples looked homogeneous, but in CE-HPLC they were found to be contaminated by β -Asp-AngI, in the range of 2.3–17%, and by various other minor contaminants; a typical elution pattern is shown in Fig. 2.

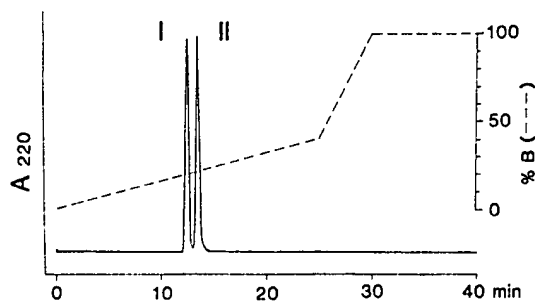


Fig. 1. Separation of β -Asp¹- and Asp¹-angiotensin II by CE-HPLC. Column: TSK gel CM-2SW (250 \times 4.6 mm I.D.). The column was equilibrated with solvent A, and then the peptide was eluted with solvent B using a gradient as shown in the figure. See text for other details. Peaks: I = β -Asp¹-AngII; II = Asp¹-AngII.

Next, we tested the homogeneity of commercial bradykinin (BK) samples since this peptide is also widely available. Only one commercial sample gave a single peak in RP-HPLC, but the shape was somewhat broader than that of our standard sample. Even the best sample gave a relatively large extra peak in CE-HPLC as shown in Fig. 3; the peak area was about 13% of the total. The structure of the component in the side peak was examined by chymotryptic mapping in HPLC and by amino acid analysis; it was confirmed to contain a normal bradykinin sequence (1–8), identical with that isolated from an authentic sample, together with one Orn residue instead of one Arg residue. Thus, the main component of the side peak was Orn⁹-BK.

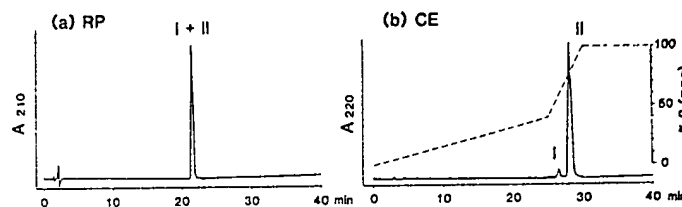


Fig. 2. RP- and CE-HPLC profiles of a commercial AngI. (a) RP-HPLC. Column: Nucleosil 5C18 (150 \times 4 mm I.D.). Gradient: 10–60% acetonitrile in 10 mM H_3PO_4 - K_2HPO_4 (pH 2.6) containing 50 mM Na_2SO_4 . (b) CE-HPLC. Column and elution conditions as in Fig. 1. Peaks: I = β -Asp¹-AngI; II = Asp¹-AngI.

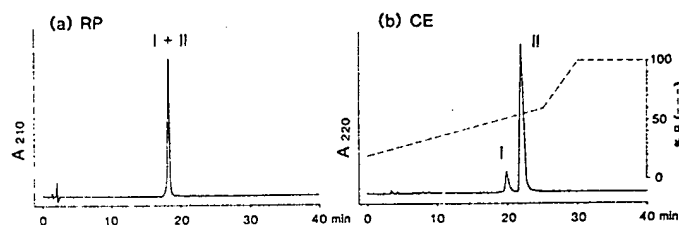


Fig. 3. RP- and CE-HPLC profiles of a commercial bradykinin. (a) RP-HPLC. Column and elution conditions as in Fig. 2. (b) CE-HPLC. Column as described in Fig. 1. It was equilibrated with 20% B in A before applying the peptide; then the peptide was eluted with solvent B using a gradient as shown. Peaks: I = Orn⁹-BK; II = BK.

Today, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system; the present results clearly indicate that the purification of synthetic peptides by RP-HPLC is not sufficient to obtain homogeneous products.

With regard to the separation of peptide diastereomers, the usefulness of RP-HPLC has been recognized by many groups, and several examples of diastereomer separation have been reported by Dizdaroglu's group^{10,11}, who used a weak anion-exchange column. We also tried to separate D-Ala⁷³,Asp⁷⁶-hPTH (39–84) from the L-peptide by our CE-HPLC, but we were unsuccessful despite the good separation of the same compounds in our RP-HPLC system⁵. A similar result was obtained with a pair of shorter peptides, D-Glu²²-hPTH (18–28) and its L-isomer. However, in the case of human growth hormone-releasing factor (hGRF), D-Leu²²-hGRF(1–44)-NH₂ was clearly separated from its original L-peptide not only by RP-HPLC¹² but also by CE-HPLC as shown in Fig. 4. From these observations, we concluded that some diastereomeric isomers can be separated by IE-HPLC but the system may not be suitable in general for detecting racemization during peptide synthesis.

Finally, we examined the separation of Asp⁷⁶-hPTH (1–84) from Asn⁷⁶-hPTH by the CE-HPLC system. Clear separation was achieved even when a 1:1 mixture was applied, as shown in Fig. 5. This CE-HPLC technique was then used to detect possible conversion of the Asn residue into Asp under the various conditions used to isolate and purify natural hPTH; that is, the peptides were kept in water, 0.1 M ammonium acetate solution (pH 2.5, 5.0 or 7.5), 0.1 M ammonium hydrogen carbonate solution (pH 8.7) or 4% TCA solution for a maximum of 5 days at room

CE = cation
exchange

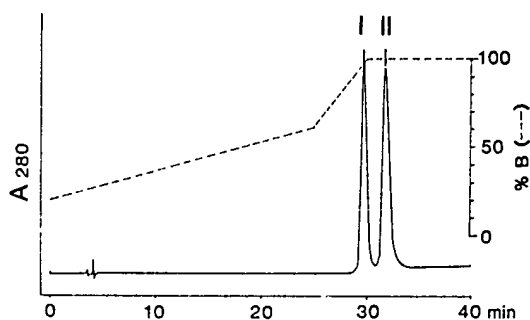


Fig. 4. Separation of D-Leu²²-hGRF(1-44)-NH₂ and the all-L-peptide by CE-HPLC. Column and elution conditions as in Fig. 3b. Peaks: I = D-Leu²²-hGRF(1-44)-NH₂; II = hGRF(1-44)-NH₂.

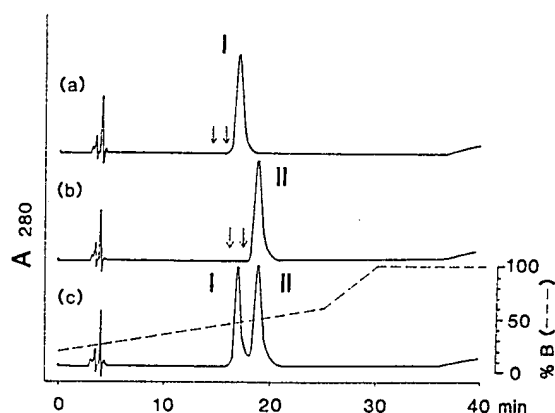


Fig. 5. Separation of Asp⁷⁶- and Asn⁷⁶-hPTH(1-84) by CE-HPLC. (a) Application of compound I only; (b) application of compound II only; (c) application of a mixture of I and II. Column and elution conditions as in Fig. 3b. Peaks: I = Asp⁷⁶-hPTH(1-84); II = Asn⁷⁶-hPTH(1-84). Arrows indicate the peak positions of Met(O)-containing peptides.

temperature¹³. No clear evidence of conversion of the Asn residue into Asp was obtained. Recently, Gleed *et al.*¹⁴ confirmed, by using radioimmunoassay, that our synthetic Asn⁷⁶-hPTH had better cross-reactivity with an antibody, raised against an isolated natural hPTH, than our Asp⁷⁶-hPTH. Furthermore, the cross-reaction curve of Asn⁷⁶-hPTH was completely parallel to that of the natural hormone. From this evidence, together with the results of the above conversion tests, we concluded that the Asp⁷⁶ structure claimed for hPTH might have been due to misreading the results of the original Edman degradation reactions.

REFERENCES

- 1 H. T. Keutmann, M. M. Sauer, G. N. Hendy, J. L. H. O'Riordan and J. T. Potts, Jr., *Biochemistry*, 17 (1978) 5723.
- 2 G. N. Hendy, H. M. Kronenberg, J. T. Potts, Jr. and A. Rich, *Proc. Nat. Acad. Sci., U.S.A.*, 78 (1981) 7365.
- 3 T. Kimura, T. Morikawa, M. Takai and S. Sakakibara, *J. Chem. Soc., Chem. Commun.*, (1982) 340.

- 4 T. Kimura, M. Takai, K. Yoshizawa and S. Sakakibara, *Biochem. Biophys. Res. Commun.*, 114 (1983) 493.
- 5 T. Kimura, K. Yoshizawa, M. Takai and S. Sakakibara, in T. Y. Liu, S. Sakakibara, A. N. Schechter, K. Yagi, H. Yajima and K. T. Yasunobu (Editors), *Frontiers in Biochemical and Biophysical Studies of Proteins and Membranes*, Elsevier, New York, 1983, pp. 81-88.
- 6 F. E. Regnier, *Anal. Biochem.*, 126 (1982) 1.
- 7 M. Dizdaroglu, in W. S. Hancock (Editor), *Handbook of the Use of HPLC for the Separation of Amino acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, pp. 23-43.
- 8 T. Kimura, M. Takai, Y. Masui, T. Morikawa and S. Sakakibara, *Biopolymers*, 20 (1981) 1823.
- 9 S. Sakakibara, H. Sokabe, T. Nakajima, M. C. Khosla, S. Kumagaye and T. X. Watanabe, in B. Lofis (Editor), *Proceedings of the Ninth International Symposium on Comparative Endocrinology*, Hong Kong University Press, Hong Kong, in press.
- 10 M. Dizdaroglu and M. G. Simic, *J. Chromatogr.*, 195 (1980) 119.
- 11 M. Dizdaroglu, M. G. Simic, R. Rioux and S. St-Pierre, *J. Chromatogr.*, 245 (1982) 158.
- 12 N. Chino, S. Kumagaye, Y. Masui, T. Kimura and S. Sakakibara, in E. Munkata (Editor), *Peptide Chemistry 1983*, Protein Research Foundation, Osaka, 1984, pp. 285-290.
- 13 H. T. Keutmann, P. M. Barling, G. N. Hendy, G. V. Segre, H. D. Niall, G. D. Aurbach, J. T. Potts, Jr. and J. L. H. O'Riordan, *Biochemistry*, 13 (1974) 1646.
- 14 J. H. Gleed, G. N. Hendy, T. Kimura, S. Sakakibara and J. L. H. O'Riordan, *J. Endocrinol.*, in press.

Total Solid-Phase Synthesis, Purification, and Characterization of Human Parathyroid Hormone-(1-84)[†]

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ABSTRACT: The complete sequence of human parathyroid hormone [Keutmann, H. T., Sauer, M. M., Hendy, G. N., O'Riordan, J. L. H., & Potts, J. T., Jr. (1978) *Biochemistry* 17, 5723] has been synthesized by the solid-phase method employing the phenylacetamidomethyl resin. Glutamine and asparagine were attached to the peptide chain by using active ester coupling, and all other amino acids were coupled by using dicyclohexylcarbodiimide. Double coupling was employed at every cycle of the synthesis, and both deprotection and coupling were monitored qualitatively. The peptide was cleaved from the resin along with the majority of the side-chain protecting groups by anhydrous hydrogen fluoride, and the formyl group from tryptophan was removed by treatment with 1 M piperidine in 8 M urea. The synthetic protein was purified by ion-exchange chromatography on CM-Sephadex, and the fraction eluting at the position of the native hormone under

identical conditions was found to have 100% of the biological activity of the native hormone. This fraction was subjected to further CM-Sephadex chromatography under identical conditions, and the final product was found to be homogeneous by amino-terminal analysis, column chromatography, disc gel electrophoresis, and isoelectric focusing. The overall yield of the pure isolated protein was 12%. The structure of the synthetic protein was verified by (1) sequence analysis using the Edman degradation procedure, (2) amino acid composition, (3) comparison of the biological activity in vitro by the adenylate cyclase assay using dog and rat renal plasma membranes, and (4) comparison of the immunological activity specific to the intact molecule as well as an assay specific for the "mid region" of the molecule with the corresponding native human and bovine hormones.

Parathyroid hormone has a major role in the modulation of the calcium concentration in blood, and the primary amino acid sequence of bovine (Brewer & Ronan, 1970; Naill et al., 1970) and human parathyroid hormone (bPTH and hPTH, respectively)¹ (Keutmann et al., 1978) has been reported. The majority of the biological and physiological studies of hPTH have been performed by utilizing synthetic fragments of the hormone, and fragments covering the complete sequence have been synthesized (Tregear et al., 1974; Rosenblatt et al., 1978). However, the total synthesis of this 84 amino acid peptide by using the solid-phase method was considered difficult because of the limitations associated with the synthesis of large peptides or proteins using the conventional Merrifield resin. A major problem with solid-phase synthesis techniques is the complexity of the final product, especially during the syntheses of long peptides (Merrifield, 1969). In addition to the incomplete reactions at various steps during the synthesis, the peptide can rearrange or cleave off from the solid support. The conventional Merrifield resin, 1% or 2% cross-linked poly(styrene-co-divinylbenzene) resin, undergoes extensive acidolysis during long peptide synthesis which reduces the overall maximum yield. As a result, attempts to synthesize larger peptides like ribonuclease A (Gutte & Merrifield, 1971) and human leukocyte interferon (Smith et al., 1981) resulted in relatively low yields. In addition to the yield, the heterogeneity of the final product frequently is significant, making it difficult to isolate the protein to homogeneity. Thus, the stability of the pep-

tide-resin bond to acidolysis is of paramount importance in solid-phase peptide synthesis.

The [[[(4-(oxymethyl)phenyl)acetamido]methyl]poly(styrene-co-divinylbenzene) resin (PAM resin) introduced by Merrifield and his co-workers (Mitchell et al., 1976) possesses increased acid stability. The electron-withdrawing phenylacetamidomethyl (PAM) group inserted between the peptide and the polystyrene matrix increases the acid stability (e.g., 50% trifluoroacetic acid in methylene chloride) of the peptide ester by 100-fold relative to the peptide ester from the conventional Merrifield resin. Use of this resin not only increases the yield but also has the added benefit of greatly reducing trifluoroacetylation, which results in chain termination. As a result, the PAM resin enhances the applicability of the solid-phase method for the synthesis of longer peptides.

We undertook the chemical synthesis of human parathyroid hormone in order to prove the stability and usefulness of the PAM resin. hPTH was selected because of its size, because it is a well-characterized protein which has been studied in some detail, and, most importantly, because it is in extremely short supply from the native source. We report here in detail the first successful solid-phase synthesis of a long polypeptide, hPTH, by using the PAM resin and its physical characteristics, biological activity, and immunological properties. In addition, the physicochemical properties of synthetic hPTH are compared to those of the native human and bovine parathyroid hormones.

Materials and Methods

The published procedures (Barany & Merrifield, 1979; Merrifield, 1969; Erickson & Merrifield, 1976) for the automated solid-phase method were followed with minor modifications. The PAM resin was prepared according to the

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¹ Abbreviations: hPTH, human parathyroid hormone; bPTH, bovine parathyroid hormone; HF, hydrogen fluoride; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; DMF, dimethylformamide; Cl-Z, α -Boc-L- α -chlorobenzyloxycarbonyl-L-lysine.

modified procedure of Tam et al. (1979). The 4-[[[(*tert*-butoxycarbonyl)glutamyl]aminoacyl]oxy]methyl]-benzeneacetic acid was prepared first in excellent yield (40%), as well as purity, and was coupled to the aminomethyl resin (1% cross-linked, 100–200 mesh) by using DCC. A relatively low substitution level of 0.2 mmol/g of resin was used in this synthesis. The excess free amino groups of the resin were blocked by acetylation by using *N*-acetylimidazole. A sample of the resin was deprotected with 50% TFA-CH₂Cl₂ and hydrolyzed in HCl-propionic acid, and the glutamic acid was quantitated by amino acid analysis to be 0.197 mmol/g of resin.

The Boc-Gln-PAM resin (3.5 g, 0.69 mM Gln) was placed in the reaction vessel of a Beckman 990B peptide synthesizer, and the instrument was programmed to perform the synthesis automatically. However, a more controlled synthesis with frequent monitoring was performed which, for all practical purposes, was performed semiautomatically. All amino acids were protected at the α -amino position with the *tert*-butoxycarbonyl group (Boc), a mild acid-labile protecting group, except for arginine, where the more soluble amyloxycarbonyl (Aoc) derivative was employed. The following side-chain protecting groups were used: Asp (OBzl), Glu (OBzl), Ser (Bzl), Thr (Bzl), Lys (Cl-Z), Arg (Tos), His (Tos), and Trp (CHO). The side chain of methionine was left unprotected. Boc or Aoc groups were removed at each cycle of the synthesis by treatment with TFA-CH₂Cl₂. Initially, a 25% solution of TFA was used, and the acid concentration was increased to 30% by step 15 and to 35% by step 25, and a concentration of 40% was used from step 40 to the end of the synthesis. A reaction time of 30 min was found to be sufficient for complete deprotection of even the sterically hindered amino acid residues in this protein.

All couplings, except for asparagine and glutamine, were performed by the dicyclohexylcarbodiimide (DCC) method, which has been used successfully for the synthesis of numerous peptides. A 4.2-mmol sample of Boc-amino acid, a 6-fold excess, in methylene chloride was added to the peptide resin followed by an equimolar amount of DCC in CH₂Cl₂. Because of their low solubility, Aoc-Arg (Tos) and Boc-His (Tos) were dissolved in a 1:1 mixture of CH₂Cl₂-DMF. The reaction time for all DCC couplings was 2 h, and double coupling was employed at every cycle. Both deprotection and coupling were qualitatively monitored by the Kaiser ninhydrin test (Kaiser et al., 1970). Boc-Asn and Boc-Gln were coupled as their *p*-nitrophenyl esters in DMF, employing a 10-fold excess of the Boc-amino acid and a reaction time of 16 h. Double coupling was also employed in these reactions, and after every second active ester coupling, the peptide resin was treated with *N*-acetylimidazole (Markley & Dorman, 1970) to block any unreacted amino group. 1,2-Ethanedithiol (1%) was added to TFA in CH₂Cl₂ during all cleavages after the first methionine was attached to the peptide resin to protect the methionine residues from any acid-catalyzed oxidation. Boc-*N*^{trp}-formyltryptophan was used in order to protect the indole nucleus of tryptophan from oxidation (Ohno et al., 1973). To avoid dilution of the acid and to swell the resin properly, we included a preliminary 2-min wash with acid before the deprotection step. The trifluoroacetate salt was neutralized by a prewash with 10% triethylamine in CH₂Cl₂ followed by two 8-min treatments with the same reagent.

After the complete primary sequence of hPTH was synthesized, the last Boc group was removed with TFA prior to the HF cleavage. The peptide was cleaved from the resin with the simultaneous removal of the side-chain protecting groups

by treatment with anhydrous HF. One gram of the protected peptide resin was treated with anhydrous HF (10 mL) in the presence of anisole (2 mL) and methyl ethyl sulfide (0.25 mL) as scavengers at -20 °C for 15 min and at 0 °C for 60 min as previously described (Sakakibara et al., 1967). Excess HF was removed under high vacuum, and the residue was washed 3 times with anhydrous ether. The peptide was extracted with aqueous acetic acid (10%) and lyophilized. The crude peptide was treated with 50 mL of 5% sodium bicarbonate in order to reverse any N \rightarrow O acyl shift, which might have occurred during the HF cleavage (Sakakibara et al., 1962). After 2 h at pH 7.5, the synthesized polypeptide was dialyzed at 4 °C against distilled water and lyophilized. The *N*^{trp}-formyl group from the tryptophan was removed by treating the protein with 1 M piperidine in 8 M urea at 0 °C for 45 min (Ohno et al., 1973).

Purification. The crude peptide was initially desalted by gel filtration on a Bio-Gel P-2 column in 0.1 M acetic acid. The fractions corresponding to the major peptides were pooled and lyophilized, and the protein was chromatographed on a CM-Sephadex ion-exchange column (13 \times 0.5 cm). The column was equilibrated with 0.05 M ammonium acetate in 6 M urea at pH 5.6 (buffer A). Crude synthetic hPTH was dissolved in 0.025 M ammonium acetate in 6 M urea and applied to the column. Initially, 30 mL of buffer A was passed through the column, followed by a gradient of 0.11–0.25 M ammonium acetate–6 M urea at a flow rate of 6 mL/h. All fractions were collected and tested for the biological activity *in vitro* by the activation of renal adenylate cyclase. Fractions showing maximal activity (90–100%) were pooled and desalted on a Bio-Gel P2 column in 0.1 M acetic acid. The synthetic peptide was further purified by two additional fractionations under similar experimental conditions which yielded a single component on CM-Sephadex C-25 chromatography. The final yield of the purified synthetic hPTH was 12%. Native human and bovine parathyroid hormones were purified by gel filtration and ion-exchange chromatography as described earlier (Brewer & Ronan, 1970; Brewer et al., 1972).

Purity Assessment. Amino acid sequence analysis of the synthesized peptide was carried out in a modified Beckman 890B peptide sequencer by using the procedure described by Fairwell & Brewer (1979). Approximately 600 nmol of the synthetic peptide was used, and the degradations were carried out for 60 cycles. The phenylthiohydantoin amino acids were identified quantitatively by high-performance liquid chromatography (HPLC) (Zimmerman et al., 1977) as well as by chemical ionization mass spectrometry (Fairwell & Brewer, 1980). A large quantity (5–10%) of the derivative of each step in the sequence was used for the HPLC analysis, which permitted the detection of a preview of the next residue of as little as 0.01%. The amino acid composition of the crude peptide as well as the purified material from the CM-Sephadex column was determined on a Beckman 120 analyzer after acid hydrolysis in constant-boiling HCl at 110 °C for 24 h in the presence of mercaptoethanol.

Polyacrylamide gel electrophoresis in the presence of 8 M urea was performed at pH 4.4 (0.6 \times 8.5 cm glass tubes) by utilizing a stacking gel (2.5% acrylamide with 7.4% cross-linkage) and a running gel (10% acrylamide with 2% cross-linkage). The anodic buffer (pH 5.3) contained 0.05 M KOH–0.06 M acetic acid in 2 M urea, and the cathodic buffer (pH 6.1) was 0.05 M β -alanine and 0.045 N acetic acid in 2 M urea. Protein samples (10–15 μ g) were dissolved in 50 μ L of the cathodic buffer containing 25% sucrose. Methyl green was used as the tracking dye. Electrophoresis was performed

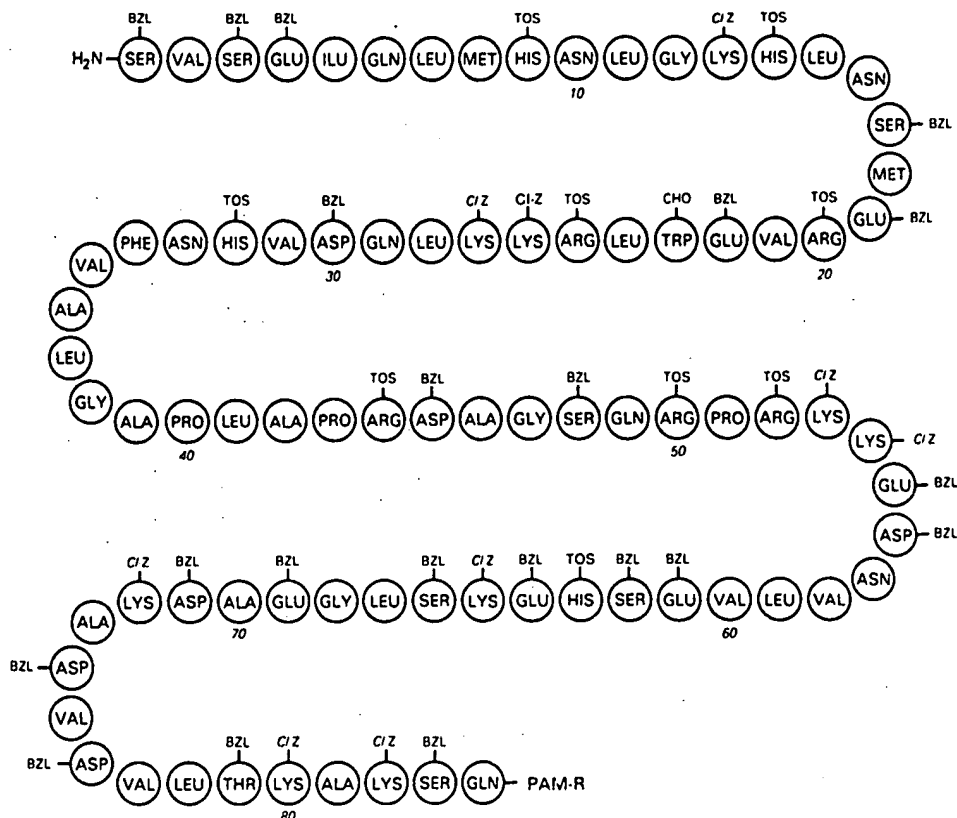


FIGURE 1: Amino acid sequence of hPTH showing the side-chain protecting groups used during the synthesis. The synthesis was started from the glutamine at the C terminal.

at 2–5 mA/tube for 2 h, and the gels were fixed with 20% trichloroacetic acid for 16 h followed by staining with 1% Coomassie blue for 6 h. Destaining was carried out with 7.5% acetic acid.

Polyacrylamide gel isoelectrofocusing was performed at a pH range of 3–10. Protein samples were isoelectrofocused in a 7.5% acrylamide disc gel (0.6 × 7.5 cm glass tubes) containing 8 M urea and 3% ampholine for 21 h by applying a constant voltage (250 V). Gels were stained with 0.2% Coomassie brilliant blue G-250 in 12% trichloroacetic acid for 16 h and destained with 7.5% acetic acid.

Bioassay and Immunoassay. Biological activity of the synthetic peptide in vitro was performed in two systems by using rat renal and dog renal plasma membranes (Krishna et al., 1968; Marcus & Aurbach, 1971; Nissensen & Arnaud, 1979). The dose-response curve of the peptide was determined in triplicate within the linear portion of the curve. An MRC standard, bovine native parathyroid hormone (MRC 72/286), with a potency of 2600 IU/mg, was used as the standard.

The immunological assay for hPTH was performed according to the procedure described by Arnaud et al. (1971). "Intact" PTH was assayed by using antiserum CM-12M. Midregion-specific immunoreactivity was assessed by using antiserum GPIM (Gallagher et al., 1980).

Results

The amino acid sequence of human parathyroid hormone with the side-chain protecting groups assembled on the PAM resin is illustrated in Figure 1. The total weight of the fully protected peptide-resin was 85% of the calculated theoretical amount, based on the amount of glutamine initially attached to the resin. This can be considered as a nearly quantitative yield, considering the fact that small aliquots of sample were

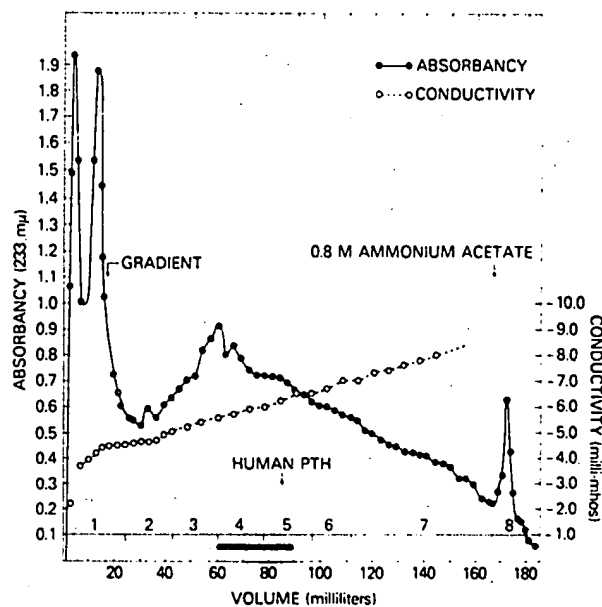


FIGURE 2: Elution profile of crude synthetic hPTH on CM-Sephadex C-25 using a linear gradient of ammonium acetate in 6 M urea. Bioassay using the in vitro dog renal adenylate cyclase assay system indicated that pools 4 and 5 contained maximal biologically active peptide (shaded area). Fractions contained in this area were pooled for further purification (see Figure 3).

removed twice at each cycle for the Kaiser ninhydrin test. Amino acid analysis of the crude peptide showed a 64% yield following HF cleavage.

The crude peptide was initially desalted by gel chromatography on Bio-Gel P-2. The major fraction was purified by

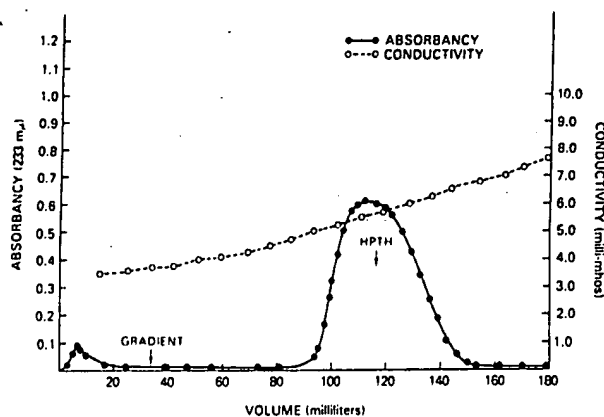


FIGURE 3: Rechromatography of biologically active synthetic hPTH on CM-Sephadex C-25. Fractions corresponding to the major peptide (100–140 mL) were pooled and desalted.

Table I: Amino Acid Composition of Purified hPTH^a

amino acid	theoretical composition	synthetic hPTH ^b
aspartic acid	10	10.2
threonine	1	0.8
serine	7	6.5
glutamic acid	11	11.1
proline	3	3.4
glycine	4	4.4
alanine	7	7.1
valine	8	7.3
methionine	2	2.0
isoleucine	1	1.0
leucine	10	10.5
phenylalanine	1	1.0
histidine	4	4.0
lysine	9	9.0
arginine	5	5.2

^a Moles of amino acid per mole of protein. ^b Duplicate values obtained from 24-h hydrolysis in 5.7 M HCl at 110 °C.

ion-exchange chromatography on a CM-Sephadex C-25 column (Figure 2). Amino acid analysis of samples taken from different fractions in the region of elution of native hPTH indicated that the desired peptide was present in the middle region of the major peak. Native human PTH eluted at a conductivity of 6.5 mΩ⁻¹ under the same chromatographic conditions. The amino acid compositions of pools 4 and 5 were in excellent agreement with the theoretical values for human PTH. Fractions 4 and 5 were pooled and rechromatographed twice on a CM-Sephadex column under identical conditions, and the final chromatographic product was a single component eluting at the same position as the native hormone under identical conditions (Figure 3). Amino acid analysis of the final product was in excellent agreement with theoretical values for the hPTH native peptide (Table I). The final yield of the purified synthetic hPTH was 12%.

Automated Edman sequence analysis of the CM-Sephadex-purified peptide for 60 cycles revealed the desired sequence with only 5–6% preview of the next residue, resulting from incomplete cleavage or coupling during the synthesis. Estimation of the deletion at any point in the desired sequence was performed as described by Tregear et al. (1974). The percentage of preview sequences observed at selected steps is shown in Table II. As is well-known in solid-phase synthesis, the deletion sequences were found after isoleucine at step 5 and at various prolines in the middle of the sequence. An additional difficult residue to couple in the synthesis is the glutamine at step 29, as reported by Tregear et al. (1974).

Table II: Preview of Amino Acid Derivatives Observed during Edman Degradation of Synthetic hPTH

sequence step no.	residue ^a	preview residue	% of preview residue
1	Ser	Val	0.0
5	Ile	Gln	0.61
10	Asn	Leu	0.85
15	Leu	Asn	0.32
20	Arg	Val	1.4
25	Arg	Lys	2.4
30	Asp	Val	3.15
35	Val	Ala	5.8
40	Pro	Leu	8.2
45	Asp	Ala	11.6
50	Arg	Pro	8.2
55	Glu	Asp	8.3
60	Val	Glu	14

^a No corrections were made for the background peaks. Previews were observed mostly after the isoleucine at step 5 and the prolines at steps 40, 43, and 51.

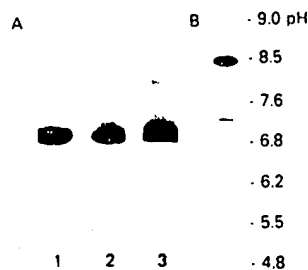


FIGURE 4: (A) Polyacrylamide gel electrophoresis (pH 4.4) of purified (1) synthetic hPTH, (2) native hPTH, and (3) native bPTH. (B) Polyacrylamide gel isoelectrofocusing of purified synthetic hPTH at a pH range of 3–10.

However, we have blocked any unreacted amino group by acetylation at this step. The preview of glutamic acid at step 60 was only approximately 7% after correction for the Glu from background peaks. This is performed by subtracting an average peak area that can be attributed to Glu from the background (as a result of problems associated with the Edman sequence analysis). In the sequence analysis, the amino acids were found to be in the correct sequence for 60 cycles, and no incomplete deprotection of the side-chain protecting groups or acylation of anisole by the carboxyl groups was observed in the mass spectra of the phenylthiohydantoin derivatives.

Polyacrylamide gel electrophoresis in 8 M urea at pH 4.4 of the synthetic peptide showed a component which was similar to the mobility of reference samples of native human and bovine parathyroid hormone (Figure 4A). Analytical isoelectric focusing revealed one major and one minor component (Figure 4B). The pI of the major component was 8.5, the same as that for the native hormone.

The approximate potencies (dog renal plasma membrane adenylate cyclase assay) of the crude peptide and the various pools from the CM-Sephadex chromatography fractionation (Figure 2) are shown in Table III. These were derived from parallel dose-response curves and are expressed as the dose of peptide producing half-maximal stimulation of adenylate cyclase in the assay. The native human hormone used in these assays was equivalent in activity to the native bovine hormone (MRC standard 72/286; potency 2600 IU/mg), and the synthetic peptide isolated in pools 4 and 5 demonstrated the greatest activity and was 95% and 100% as active, respectively, as the preparation of native hPTH used as standard in all assays. There were no differences in the potencies of native hPTH, native bPTH, and synthetic hPTH by using dog renal

Table III: Biological Activity of Synthetic hPTH^a

sample	ng of peptide for half-max activity	% of native hPTH- (1-84)
native hPTH-(1-84)	100	
crude synthetic hormone	400	24
CM-Sephadex: pool 1 ^b	2000	5
pool 2	200	50
pool 3	180	60
pool 4	110	95
pool 5	100	100
pool 6	150	75
pool 7	200	50
pool 8	300	33

^a Biological activity determined by adenylate cyclase assay in dog renal plasma membranes. ^b See Figure 2 for elution positions of various pools.

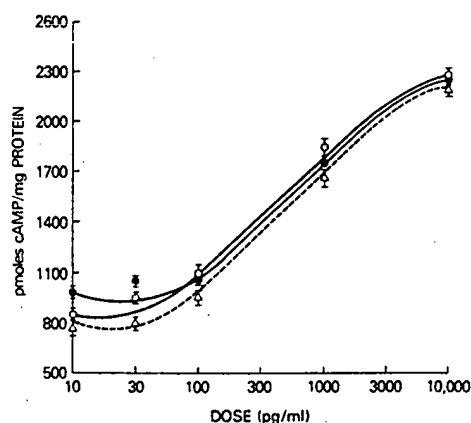


FIGURE 5: Comparison of dose-response curves for purified native hPTH (●), native bPTH (○), and synthetic hPTH (Δ) in dog renal adenyl cyclase assay systems. Incubation time was 30 min at 30 °C. (The bovine native PTH employed is an MRC standard with a potency of 2600 IU/mg.) Each point is the mean \pm SE of triplicate determinations.

plasma membranes (Figure 5). However, the potencies of native and synthetic hPTH's were approximately 3 times lower than that for native bPTH by using rat renal cortical membranes (data not shown).

Comparison of the immunologic reactivity of our synthetic preparation of hPTH with those of native bPTH and hPTH in radioimmunoassays specific for intact hPTH and mid-region hPTH is shown in panels A and B, respectively, of Figure 6. Although the immunopotency of the synthetic peptide is similar to that of the native peptides in both assay systems, the slopes and configurations of the dose-response curves for the synthetic peptide appear to differ slightly from those of the native peptides.

Discussion

The successful synthesis of human parathyroid hormone-(1-84) by the solid-phase technique required that the resin used as the solid support be stable during the acidolytic deprotection of Boc groups from the amino terminus of the assembled peptide. For this reason, the PAM resin was chosen with a low substitution level. The synthesis procedure described by Tam et al. (1979) allows exact control of the extent of amino acid substitution, and the free amino groups of the resin can be blocked by acetylation, preventing any undesirable side reactions. With the growing chain length of the peptide, the volume of the resin increased; however, its physical characteristics were not noticeably changed. The stability and

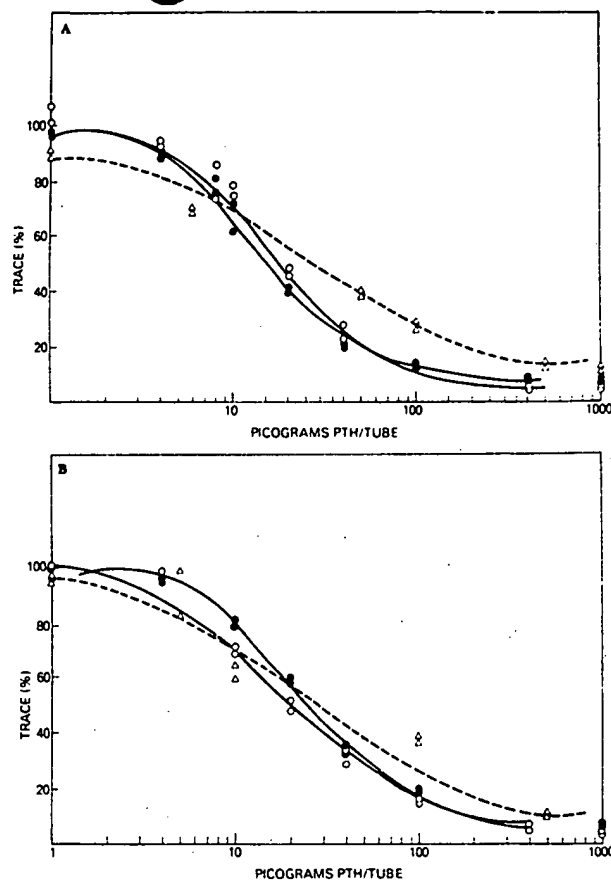


FIGURE 6: Radioimmunoassay dose-response curves for bPTH (○), native hPTH (●), and synthetic hPTH (Δ) using CH 12M antiserum (A) (specific for intact hPTH) and GP 1M antiserum (B) (specific for mid-region hPTH).

the behavior of this solid support were clearly of great importance in the solid-phase peptide synthesis. Side-chain protecting groups for the individual amino acid chains were selected so that they were relatively stable toward the reagents used and at the same time could be removed in one step together with the cleavage of the peptide from the solid support, except for tryptophan, where the formyl group from the indole nucleus was removed by piperidine.

The reaction conditions, 6-fold excess of the amino acid and DCC, and the double coupling at every cycle were all chosen for this synthesis in order to achieve the maximum yield and reduce the quantity of other incomplete peptides. The individual deprotection and coupling steps were qualitatively monitored even though it necessitated the use of the synthesizer in a semiautomatic manner. An initial acid concentration of 25% TFA was used to deprotect the α -amino groups, and the acid concentration was raised to 40% by step 40. Even though the ester bond attaching the peptide chain to the resin was extremely stable toward 50% TFA in CH_2Cl_2 as reported by Merrifield and his co-workers (Mitchell et al., 1976), it has been proposed that the benzyl ester group used to protect the side-chain functions of various amino acid derivatives can be better protected by reducing the acid concentration. As the peptide grows longer in length, steric factors can influence the extent of deprotection at every stage, and, therefore, the acid concentration was increased as the synthesis progressed. A final concentration of 40% TFA was used from cycle 40 to the completion of the synthesis. It is difficult to completely ascertain the effectiveness of this procedure, and it is probably

not required when small peptides are synthesized. Even at the low acid concentration (25%), the 30 min used for deprotection should be more than adequate to deprotect even the sterically hindered amino acid derivatives. There is, however, no definite data to demonstrate that these changes in the acid concentration reduced the heterogeneity of the final product. Methionine-containing peptides are known to undergo *tert*-butylation, during HF cleavage, when the peptides have been protected with Boc groups, and in this synthesis, the last Boc group was removed before HF cleavage. Edman sequence analysis has been reported to be a very sensitive and convenient method for the detection of deletion sequences (Tregear et al., 1974). In our opinion, it is desirable to use a large quantity of the peptide and identify the amino acid derivatives representing the deletion peptides. In most cases, by overloading the HPLC column, it is possible to detect as low as 0.01% of a deletion sequence. However, it is difficult to estimate the exact percentage of preview toward the end of a long sequence run, because of the fact that the number and quantity of background peaks due to nonspecific cleavage of the peptide bonds as well as the incomplete coupling and cleavage during the sequence analysis increase, making it difficult to ascertain what percentage of a particular peak is due to preview. However, a significant quantity of a deletion sequence at any step can be easily detected. Mass spectral analysis of these derivatives can be used to detect incomplete removal of side-chain protecting groups as well as acylation of anisole by the carboxyl groups of the peptide. The limitations of each of these methods are due to inherent problems in the chemistry of the Edman reaction itself, and the analysis of derivatives near the end of a 60-cycle sequence run may only be useful for determining the primary sequence of the peptide. The purified synthetic peptide had the desired sequence for the first 60 residues from the amino terminus. Since the 53–84 region of this peptide has been synthesized without any difficulty and no serious problems were noticed, it is only logical to assume that any deletion or chain termination which occurred during the synthesis could easily be detected by the sequence analysis of the amino-terminal end. The overall amino acid composition of the synthetic material was identical with that of the native hormone. The synthetic peptide chromatographed in the position of the native human hormone on CM-Sephadex, and the purified synthetic hormone possessed nearly 100% biological activity in adenyl cyclase assays when compared with native hPTH. The difference in the potency of the native human and bovine hormones in the rat assay may be attributed to the specificity of the two membrane species used in the assays. Previous studies in several laboratories have shown that deletions in the amino-terminal sequence of PTH are associated with reductions in biological activity.

The only evidence that we have been able to obtain to date suggesting that the hPTH we have synthesized might be different from the native hPTH is immunologic. The shapes and slopes of the dose-response curves produced by the synthetic peptide in two radioimmunoassays with different specificities for hPTH demonstrate minor differences when compared with those of native bPTH and hPTH. Whether these differences reflect an artifact of our radioimmunoassay systems or an alteration from the true structure of the native hormones is conjectural at present, and the problem requires further study. One possible explanation is that the peptide we have synthesized contains an Asp residue in position 76 as first reported by Keutmann et al. (1978) whereas this position has been reported to be Asn by Hendy et al. (1981), who determined the structure by nucleotide sequencing. Similar im-

munoassay results were reported by Manning et al. (1981) using human PTH antiserum. The synthetic C-terminal peptide (53–84 with Asp at position 76) was reported to be less reactive than the corresponding native hormone. We are currently synthesizing hPTH with Asn at position 76, and it may be possible to clarify this apparent immunological difference by investigating the immunological behaviors of the Asp- and Asn-containing peptides as compared with native hPTH.

The yield of 12% hPTH in the present synthesis was excellent, considering the size of this protein. Moreover, it is reasonable that, with optimization of reaction conditions, the yield will improve and the time required for the synthesis will be shortened.

Clearly, the scarcity of hPTH obtained from natural sources has been an important impediment to progress in the study of the physiology and pathophysiology of mineral and bone metabolism in humans. The availability of synthetic hPTH should now permit detailed clinical and biochemical studies of the role of this important hormone in calcium metabolism.

Registry No. Human parathyroid hormone-(1–84), 68893-82-3.

References

- Arnaud, C. D., Tsao, H. S., & Littledike, E. T. (1971) *J. Clin. Invest.* 50, 21.
- Barany, G., & Merrifield, R. B. (1979) in *The Peptides* (Gross, E., & Meienhofer, J., Eds.) Vol. 2, pp 1–208, Academic Press, New York.
- Brewer, H. B., Jr., & Ronan, R. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 67, 1862.
- Brewer, J. B., Jr., Fairwell, T., Ronan, R., Sizemore, G. W., & Arnaud, C. D. (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 3585.
- Erickson, B. W., & Merrifield, R. B. (1976) *Proteins (3rd Ed.)* 2, 255–527.
- Fairwell, T., & Brewer, H. B., Jr. (1979) *Anal. Biochem.* 99, 242.
- Fairwell, T., & Brewer, H. B., Jr. (1980) *Anal. Biochem.* 107, 140.
- Gallagher, B., Riggs, B. L., Jernbak, C. M., & Arnaud, C. D. (1980) *J. Lab. Clin. Med.* 95, 373.
- Gutte, B., & Merrifield, R. B. (1971) *J. Biol. Chem.* 246, 1922.
- Hendy, G. W., Kronenberg, H. M., Potts, J. T., Jr., & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 7365.
- Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem.* 34, 595.
- Keutmann, H. T., Sauer, M. M., Hendy, G. M., O'Riordan, J. L. H., & Potts, J. T., Jr. (1978) *Biochemistry* 17, 5723.
- Krishna, G., Weiss, B., & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther.* 163, 379.
- Manning, R. M., Adami, S., Papapoulos, S. E., Gleed, J. H., Hendy, G. N., Rosenblatt, M., & O'Riordan, J. L. H. (1981) *Clin. Endocrinol. (Oxford)* 15, 439.
- Marcus, R., & Aurbach, G. D. (1971) *Biochim. Biophys. Acta* 242, 410.
- Markley, L. D., & Dorman, L. C. (1970) *Tetrahedron Lett.* 1787.
- Merrifield, R. B. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol.* 32, 221.
- Mitchell, A. R., Erickson, B. W., Ryabsfer, N. N., Hodges, R. S., & Merrifield, R. B. (1976) *J. Am. Chem. Soc.* 98, 7357.
- Niall, H. D., Keutmann, H. T., Sauer, R. T., Hogan, M. L., Dawson, B. F., Aurbach, G. D., & Potts, J. T., Jr. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* 351, 1586.

- Nissensen, R. A., & Arnaud, C. D. (1979) *J. Biol. Chem.* 254, 1669.
- Ohno, M., Tsukamoto, S., Makisumi, S., & Izumiya, N. (1973) *Bull. Chem. Soc. Jpn.* 46, 3280.
- Rosenblatt, M., Segre, G. V., Tregear, G. W., Shephard, G. L., Tyler, G. A., & Potts, J. T., Jr. (1978) *Endocrinology (Philadelphia)* 103, 978.
- Sakakibara, S., Shin, K. H., & Hess, G. P. (1962) *J. Am. Chem. Soc.* 84, 4921.
- Sakakibara, S., Shiminishi, Y., Kishida, Y., Okada, M., & Sugihara, H. (1967) *Bull. Chem. Soc. Jpn.* 40, 2164.
- Smith, M. E., Komoriya, A., & Anfinsen, C. B. (1981) *Biology of Interferon Systems* (Demeyer, E., Ed.) p 39, Elsevier, Amsterdam.
- Tam, J. P., Kent, S. B. H., Wong, T. W., & Merrifield, R. B. (1979) *Synthesis* 12, 955.
- Tregear, G. W., van Rietschoten, J., Greene, E., Niall, H. D., Keutmann, H. T., Parsons, J. A., O'Riordan, J. L. H., & Potts, J. T., Jr. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* 355, 415.
- Zimmerman, C., Pisano, J. J., & Appella, E. (1977) *Anal. Biochem.* 77, 569.

Reactions of Spinach Ribulose-1,5-bisphosphate Carboxylase with Tetranitromethane[†]

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ABSTRACT: Tetranitromethane [TNM, C(NO₂)₄] rapidly inactivated spinach ribulosebisphosphate carboxylase (RuBPCase). The extent of inactivation was increased by preincubation of the enzyme with Mg²⁺ and bicarbonate. Activity was substantially protected by 6-phosphogluconate, a competitive inhibitor of carboxylation, by ribulose 1,5-bisphosphate, a substrate, and by *p*-(chloromercuri)benzoate, which was used to covalently block enzyme sulfhydryl groups. Sulfhydryl titration with Ellman's reagent showed that over one-third of the titratable cysteine residues were lost upon complete inactivation. The losses of activity and of sulfhydryl groups titratable in nondenatured enzyme occurred with similar kinetics. Approximately half the sulfhydryl groups that were lost upon TNM-induced inactivation were restored by mercaptoethanol and dithiothreitol (DTT). High concentrations

of DTT, however, did not restore more than 20% of the activity lost. Amino acid analysis revealed that about one-third of the modified sulfhydryls had been irreversibly oxidized. Both amino acid analysis and difference spectroscopy showed that little or no tyrosine modification occurred. These results suggest that TNM inactivates spinach RuBPCase by modification of cysteine sulfhydryls. This indicates that the inactivation of RuBPCase associated with SH modification does not depend solely on the presence of bulky groups attached to the modified sulfur. This conclusion differs from that obtained in similar studies using the RuBPCase of *Rhodospirillum rubrum*, in which TNM modified a single tyrosine per dimer of catalytic subunits [Robison, P. D., & Tabita, F. R. (1979) *Biochem. Biophys. Res. Commun.* 88, 85].

The enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] (RuBPCase¹) is bifunctional. It catalyzes the CO₂-fixation step in the C-3 photosynthetic carbon-reduction cycle, which converts a molecule each of D-ribulose 1,5-bisphosphate (RuBP) and CO₂ to two molecules of D-3-phosphoglycerate (3-PGA) (Calvin et al., 1955). The enzyme also catalyzes the reaction of RuBP with O₂ to yield a molecule each of 3-PGA and 2-phosphoglycolate, a substrate of photosynthetic carbon oxidation (Ogren & Bowes, 1971).

In all higher plants, the enzyme is composed of nonidentical large (*M*_r 56 000) and small (*M*_r 14 000) subunits (Siegel et al., 1972). Small subunits are not found in RuBPCase from the purple, nonsulfur bacterium *Rhodospirillum rubrum*. This enzyme consists of a dimer of large (*M*_r 56 000) subunits. RuBPCases in higher plants show great structural homology, especially in the large subunits, which bear the catalytic site (McIntosh et al., 1980; Zurawski et al., 1981). Amino acid composition data (Takabe & Akazawa, 1975a) and partial

sequence data (Stringer et al., 1981; Herndon et al., 1982) suggest that only limited sequence homology exists between RuBP carboxylase from a higher plant, spinach, and that from *R. rubrum*.

Nevertheless, affinity-labeling techniques have indicated conservation of primary structure in the active sites of carboxylases from these two sources (Herndon et al., 1982). In both enzymes, lysine residues are essential for catalysis (Paech & Tolbert, 1978; Schloss et al., 1978a; Robison et al., 1980; Lorimer, 1981b) and for the binding of activating CO₂ and Mg²⁺ (O'Leary et al., 1979; Lorimer, 1981a). Arginine (Schloss et al., 1978b; Chollet, 1981) and histidine (Saluja & McFadden, 1982) may also be important in catalysis by the spinach enzyme. Recently, Fraij & Hartman (1982) suggested that methionine is located at the active site of the *R. rubrum* enzyme. Numerous studies have shown sulfhydryl

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¹ Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; RuBPCase, ribulosebisphosphate carboxylase; PCMB, *p*-(chloromercuri)benzoate; DTT, dithiothreitol; NaDodSO₄, sodium dodecyl sulfate; PGA, 3-phosphoglycerate; TNM, tetranitromethane, C(NO₂)₄; 6-PGluA, 6-phosphogluconate; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SCM-cysteine, S-(carboxymethyl)cysteine; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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(54) **Process for the production of human parathyroid hormone**

(57) A process for the production of human parathyroid hormone (hPTH), comprising *in vivo* multiplication of

human lymphoblastoid cells capable of producing said hormone, using a non-human warm-blooded animal, and *in vitro* cultivation of the multiplied human lymphoblastoid cells to produce hPTH.

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SPECIFICATION

Process for the production of human parathyroid hormone

The present invention relates to a process for
5 the production of human parathyroid hormone
(abbreviated as hPTH hereinafter).

hPTH is a hormone, secreted by the parathyroid
glands, that regulates the synthesis of activated
vitamin D₃, stimulates the release of calcium from
10 bones and leads to an increase in the calcium level
of the blood. No process for the mass production
of low-cost hPTH has been established to date.

We have investigated processes for the mass
production of hPTH and have unexpectedly found
15 that certain human lymphoblastoid cells capable
of producing hPTH are suitable for the mass
production of hPTH owing to their very high
multiplication efficiency and high rate of hPTH
production per cell.

20 According to the present invention there is
provided a process for the production of hPTH,
which process comprises multiplying human
lymphoblastoid cells capable of producing said
hormone by transplanting said cells to a non-
25 human warm-blooded animal body, or
alternatively by allowing said cells to multiply
within a device in which the nutrient body fluid of
a non-human warm-blooded animal is supplied to
said cells, and allowing the human lymphoblastoid
30 cells multiplied by either of the above
multiplication procedures to release said hormone.

The process according to the invention provides
an extremely high hPTH yield, requires much less
nutrient medium containing expensive serum for
35 the cell multiplication or no such medium, and
renders much easier the maintenance of the
culture medium during the cell multiplication than
in *in vitro* tissue culture. Particularly, any human
lymphoblastoid cells capable of producing hPTH
40 can be multiplied easily while utilising the nutrient
body fluid supplied from the non-human warm-
blooded animal body by transplanting said cells to
the animal body, or suspending the cells in a
conventional diffusion chamber devised to receive
45 the nutrient body fluid of the animal, and feeding
the animal in the usual way. Also, in the present
process one obtains a more stable and a higher
rate of cell multiplication, and a higher hPTH
production per cell.

50 As regards the human lymphoblastoid cells
which may be used in the present invention, any
human lymphoblastoid cells can be used as long
as they produce hPTH and multiply rapidly in the
non-human warm-blooded animal body.
55 Preferable human lymphoblastoid cells are those
introduced with hPTH production governing
genetic sites of human cells which inherently
produce hPTH such as normal or transformed
parathyroid cells, or human cells which produce
60 ectopic hPTH such as lung carcinoma cells,
ovarian tumor cells, kidney carcinoma cells or liver
carcinoma cells by means of cell fusion using
polyethylene glycol, or by genetic recombination
techniques using DNA ligase, nuclease and DNA

65 polymerase; and other human lymphoblastoid
cells which produce ectopic hPTH. Since the use
of such human lymphoblastoid cells results in the
formation of easily disaggregatable massive
tumors when the cells are transplanted to the
70 animal body, and the massive tumors are barely
contaminated with the host animal cells, the
multiplied live human lymphoblastoid cells can be
harvested easily.

Any non-human warm-blooded animal can be
75 used to perform the process of the present
invention as long as the human lymphoblastoid
cells multiply therein. Examples of suitable
animals are poultry such as chickens or pigeons,
and mammals such as dogs, cats, monkeys, goats,
80 pigs, cows, horses, guinea pigs, rats, hamsters,
mice and nude mice. Since transplantation of the
human lymphoblastoid cells gives rise to
undesirable immunoreactions, the use of a
newborn or infant animal, or of an animal in the
85 youngest possible stage, for example, in the form
of an egg, embryo or foetus, is desirable. In order
to reduce the incidence of immunoreactions as
much as possible, prior to the cell transplantation
the animal may be treated with X-ray or γ -ray
90 irradiation, at about 200—600 rem, or with an
injection of antiserum or an immunosuppressive
agent prepared according to conventional
methods. Nude mice exhibit weak
immunoreactions; consequently, any human
95 lymphoblastoid lines capable of producing hPTH
can advantageously be transplanted into, and
rapidly multiplied in, nude mice without subjecting
the mice to a pretreatment for suppressing
immunoreactions.

100 Stabilised cell multiplication and enhancement
of hPTH production can be both carried out by
repeated transplantation using combination(s) of
different non-human warm-blooded animals; the
objectives are attainable first by implanting said
105 cells in hamsters and multiplying the cells therein,
then by reimplanting the cells in nude mice.
Repeated transplantation may be carried out with
animals of the same class or division as well as
those of the same species or genus.

110 The human cells to be multiplied can be
implanted in any site of the animal as long as they
multiply at that site; for example, in the allantoic
cavity, or intravenously, intraperitoneally, or
subcutaneously.

115 In addition to the above-mentioned direct cell
transplantation, any conventional human
lymphoblastoid lines capable of producing hPTH
can be multiplied easily by using the nutrient body
fluid supplied from the animal body by embedding,
120 for example, intraperitoneally, in the animal body a
conventional diffusion chamber, of various shapes
and sizes, and equipped with a porous membrane
filter, ultra filter or hollow fiber having a pore size
of about 10^{-7} to 10^{-8} m in diameter which
125 prevents ingress of the host animal cells into the
chamber but permits the cells to be supplied with
the nutrient body fluid of the animal. Furthermore,
the diffusion chamber can be designed, if desired,
so as to enable observation of the cell suspension

in the chamber through transparent side window(s) provided on the chamber wall(s), and so as to enable replacement and exchange with a fresh chamber. In this way cell production per host

- 5 can be increased to even higher levels over the period of the animal's life without any sacrifice of the host animal. When such a diffusion chamber is used, since little immunoreaction arises owing to the absence of direct contact of the human cells with the host animal cells, the multiplied human lymphoblastoid cells can be harvested easily, and any non-human warm-blooded animal can be used as the host in the present process without the need for any pretreatment to reduce
- 10 immunoreactions.

Feeding of the host animal can be carried out by conventional methods even after cell transplantation, and no special care is required.

- Maximum cell multiplication can be attained within 1—20 weeks, generally 1—5 weeks, after the cell transplantation.

- According to the invention, the number of the human lymphoblastoid cells obtained per host ranges from about 10^7 to 10^{12} or more. In other words, the number of the human lymphoblastoid cells implanted in the animal body increases about 10^2 to 10^7 times or more, or about 10^1 to 10^6 times or more than that attained by *in vitro* tissue culture method using a nutrient medium; the human lymphoblastoid cells can therefore be used advantageously in the production of hPTH.

- As regards the method by which the human lymphoblastoid cells multiplied by either of the above described procedures are allowed to release hPTH, any methods can be employed as long as the said human cells release the desired hormone thereby. For example, human lymphoblastoid cells, obtained by multiplying in ascite in suspension and harvesting from said ascite, or by extracting the massive tumor formed subcutaneously and harvesting after the disaggregation of the massive tumor, are suspended to give a cell concentration of about 10^4 to 10^8 cells per ml in a nutrient medium, prewarmed at a temperature of about 20—40°C, and then incubated at this temperature for about 1 to 100 hours to produce hPTH. During the incubation, enhancement of hPTH production may be carried out by including one or more of an amino acid such as glycine, leucine, lysine, arginine and cysteine; an inorganic salt such as sodium chloride, potassium chloride, calcium chloride and magnesium sulfate; and a hormone such as dopamine, isoproterenol, epinephrine and norepinephrine.

- The hPTH thus obtained can be collected easily by purification and separation techniques using conventional procedures such as salting-out, dialysis, filtration, centrifugation, concentration, and lyophilisation. If a more highly purified hPTH preparation is desirable, a preparation of the highest purity can be obtained by the above-mentioned techniques in combination with other conventional procedures such as adsorption and desorption with ion exchange, gel filtration, affinity chromatography, isoelectric point fractionation

and electrophoresis.

- The hPTH preparation thus obtained can be used advantageously alone or in combination with one or more agents for injection, or for external, internal or diagnostical administration in the prevention and treatment of human diseases.

The following Examples illustrate the present invention.

- In this specification, hPTH production was determined by a bioassay method as described in J. A. Parsons *et al.*, *Endocrinology*, Vol 92, pp. 454—462 (1973), and is expressed by weight in terms of the standard hPTH preparation, assigned 1,300 USP units per mg, available from the National Institute of Health (USA).

EXAMPLE 1

- Disaggregated human parathyroid tumor cells — extracted from a patient suffering from parathyroid tumor and minced — and a human leukemic lymphoblastoid line Namalwa were suspended together in a vessel with a salt solution, containing 140 mM NaCl, 54 mM KCl, 1 mM NaH_2PC_4 and 2 mM CaCl_2 , to give a respective cell concentration of about 10^4 cells per ml. The ice-chilled cell suspension was mixed with a fresh preparation of the same salt solution containing UV-irradiation preinactivated Sendai virus, transferred into a 37°C incubator five minutes after the mixing, and stirred therein for 30 minutes to effect cell fusion, thereby introducing the ability of the human parathyroid tumor cells of producing hPTH into the human leukemic lymphoblastoid line.

- After cloning according to conventional methods the hybridoma cell strain capable of producing hPTH, the hybridoma cells strain was implanted intraperitoneally in adult nude mice which were then fed in the usual way for five weeks. The resulting massive tumors, about 15 g each, were extracted and disaggregated by mincing and trypsinizing.

- After washing the cells with Earle's 199 medium (pH 7.2), supplemented with 10 v/v % foetal calf serum, the cells were resuspended to give a cell concentration of about 10^5 cells per ml in a fresh preparation of the same medium which contained 30 mM L-arginine and 20 mM CaCl_2 , and then incubated at 37°C for 40 hours to produce hPTH. Thereafter, the cells were treated ultrasonically, and the hPTH in the resulting supernatant was determined. The hPTH production was about 830 ng per ml cell suspension.

- Control cells were obtained by cultivating *in vitro* the human parathyroid cells at 37°C in Earle's 199 medium (pH 7.2), supplemented with 10 v/v % foetal calf serum. These cells were treated similarly as described above to produce hPTH. The hPTH production was only about 4 ng per ml cell suspension.

EXAMPLE 2

Disaggregated human kidney carcinoma cells — extracted from a patient suffering from

kidney carcinoma and minced — and a human leukemic lymphoblastoid line JBL were fused in a manner similar to that described in Example 1, thereby introducing the ability of the human kidney carcinoma cells of producing hPTH into the human leukemic lymphoblastoid line.

After cloning according to conventional methods the hybridoma cell strain capable of producing hPTH, the hybridoma cell strain was implanted subcutaneously in newborn hamsters which had been preinjected with an antiserum (prepared from rabbits using conventional methods) so as to reduce the immunoreactions of the rabbits. The rabbits were then fed in the usual way for three weeks.

The resulting massive tumors, formed subcutaneously and about 10 g each, were extracted and disaggregated by mincing and suspending in a physiological saline solution containing collagenase.

After washing the cells with Eagle's minimal essential medium (pH 7.4), supplemented with 5 v/v % human serum, the cells were resuspended to give a cell concentration of about 10^6 cells per ml in a fresh preparation of the same medium which contained 20 mM CaCl_2 and 20 mM dopamine, and then incubated at 37°C for 20 hours to produce hPTH. The hPTH production was about 1.3 μg per ml cell suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line JBL. The control cells were treated similarly as described above. The hPTH production was only about 16 ng per ml cell suspension.

EXAMPLE 3

Newborn rats were implanted intravenously with a human leukemic lymphoblastoid line BALL-1 into which the ability of human ovarian tumor cells of producing hPTH had been introduced in a manner similar to that described in Example 1, and then fed in the usual way for four weeks.

The resulting massive tumors, about 30 g each, were extracted and treated similarly as described in Example 1 to produce hPTH. The hPTH production was about 900 ng per ml suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line BALL-1. These control cells were treated similarly as described above. The hPTH production was only about 10 ng per ml cell suspension.

EXAMPLE 4

After about 400 rem X-ray irradiation of adult mice to reduce their immunoreactions, the mice were implanted subcutaneously with a human leukemic lymphoblastoid line NALL-1 into which the ability of human lung carcinoma cells to produce hPTH had been introduced in a manner similar to that described in Example 1. The mice were then fed in the usual way for three weeks.

The resulting massive tumors, termed subcutaneously and about 15 g each, were

extracted and treated similarly as described in Example 2 to produce hPTH. The hPTH production was about 1.2 μg per ml cell suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line NALL-1. The control cells were treated similarly as described above. The hPTH production was only about 20 ng per ml cell suspension.

EXAMPLE 5

A human leukemic lymphoblastoid line TALL-1 into which the ability of the human parathyroid tumor cells to produce hPTH had been introduced in a manner similar to that described in Example 1 was suspended in physiological saline solution, and the resulting cell suspension was transferred into a plastic cylindrical diffusion chamber (inner volume: about 10 ml) and equipped with a membrane filter having a pore size of about 0.5 μ in diameter. After intraperitoneal embedding of the chamber into an adult rat, the rat was fed in the usual way for four weeks, and the chamber was removed.

The human lymphoblastoid cell density in the chamber attained by the above operation was about 6×10^5 cells per ml which was about 10^2 times higher or more than that attained by *in vitro* cultivation using a CO_2 incubator. The human lymphoblastoid cells thus obtained were treated similarly as described in Example 2 to produce hPTH. The hPTH production was about 1.1 μg per ml cell suspension.

Control cells were obtained by suspending the human parathyroid tumor cells in physiological saline solution, transferring the resulting cell suspension in the chamber, embedding intraperitoneally the chamber into an adult rat, feeding the rat in the usual way for four weeks, and harvesting the multiplied human lymphoblastoid cells (human cell density, about 8×10^6 cells per ml). The control cells were treated similarly as described above. The hPTH production was only about 3 μg per ml cell suspension.

EXAMPLE 6

A human leukemic lymphoblastoid line JBL into which the ability of the human lung carcinoma cells to produce hPTH had been introduced in a manner similar to that described in Example 1 was implanted in the allantoic cavities of embryonated eggs which had been preincubated at 37°C for five days. After further incubation of the eggs at this temperature for an additional one week, the chamber was removed.

The multiplied human lymphoblastoid cells thus obtained were treated similarly as described in Example 1 to produce hPTH. The hPTH production was about 700 ng per ml cell suspension.

In a control experiment in which the human lung carcinoma cells were implanted in the allantoic cavities of embryonated eggs, no cell multiplication was observed.

CLAIMS

1. A process for the production of human parathyroid hormone (hPTH), which process comprises multiplying human lymphoblastoid cells
5 capable of producing said hormone by transplanting said cells to a non-human warm-blooded animal body, and allowing the multiplied human lymphoblastoid cells to release said hormone; or multiplying human lymphoblastoid
10 cells capable of producing said hormone by allowing said cells to multiply within a device in which the nutrient body fluid of a non-human warm-blooded animal is supplied to said cells, and allowing the multiplied human lymphoblastoid
15 cells to release said hormone.
2. A process according to Claim 1, wherein the human lymphoblastoid cells are hybridoma cells obtained by cell fusion of a human lymphoblastoid line with human cells capable of producing hPTH.
- 20 3. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human parathyroid tumor cells.
4. A process according to Claim 2, wherein the
25 hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human kidney tumor cells.
5. A process according to Claim 2 wherein the
30 hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human ovarian tumor cells.
6. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human lung carcinoma cells.
- 35 7. A process according to any one of Claims 2 to 6 wherein the human lymphoblastoid line is a human leukemic lymphoblastoid line.
8. A process according to any one of Claims 2
40 to 7, wherein the human lymphoblastoid line is Nalmwa, BALL-1, NALL-1, TALL-1 or JBL.
9. A process according to any one of the preceding Claims, wherein the multiplied human lymphoblastoid cells are allowed to release hPTH
45 in the presence of one or more of glycine, leucine, lysine, arginine, cysteine; sodium chloride, potassium chloride, calcium chloride, magnesium sulfate; dopamine, isoproterenol, epinephrine and norepinephrine.
- 50 10. A process according to any one of the preceding claims, wherein the non-human warm-blooded animal is a chicken, pigeon, dog, cat, monkey, goat, pig, cow, horse, guinea pig, rat, hamster, mouse or nude mouse.
- 55 11. A process according to Claim 1 substantially as hereinbefore described in any one of the Examples.
12. Human parathyroid hormone whenever prepared by a process as claimed in any one of the
60 preceding claims.

Abstracts of the Third Annual Scientific Meeting of the American Society for Bone and Mineral Research

**Netherlands Hilton Hotel, Cincinnati, Ohio
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STRUCTURAL ANALYSIS OF THE HUMAN PARATHYROID HORMONE GENE. Henry M. Kronenberg, Thomas J. Vasicsek*, Geoffrey N. Hendy*, Alexander Rich*, and John T. Potts, Jr., Endocrine Unit, Massachusetts General Hospital, Boston, MA., and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA.

We are comparing the structures of the gene and the messenger RNA (mRNA) coding for human preproparathyroid hormone (preproPTH). This comparison will allow us to define sequences controlling the initiation and termination of RNA transcription, and will also allow us to locate so-called intervening DNA sequences; that is, sequences of DNA that interrupt the PTH mRNA and are removed from an mRNA precursor before mRNA is released from the cell nucleus. The human preproPTH gene was isolated from a lambda phage human gene library. We have subcloned portions of the 14,000 base pair human DNA into the plasmid pBR322 in order to facilitate sequence analysis. We used the agarose gel transfer method of Southern to identify fragments of the human DNA containing PTH information. Human DNA fragments were separated by agarose gel electrophoresis and then transferred to nitrocellulose paper. PTH-related fragments were recognized by hybridizing the nitrocellulose-bound DNA to radioactive DNA derived from a clone containing human PTH mRNA sequences. The relevant fragments were then re-cloned into pBR322. Our analysis so far shows that no intervening sequence interrupts the gene in a 215 pair region extending from amino acid 28 of PTH through the rest of the coding region and including the first 38 nucleotides of the 3'-noncoding region of the mRNA. Since intervening sequences often separate functionally important domains, we might have expected an intervening sequence separating the biologically active 1-34 region from the distal half of PTH. Further restriction endonuclease and DNA sequence analysis will allow us to complete the characterization of the gene.

HUMAN PARATHYROID CARCINOMA CELLS THAT PRODUCE PTH: LONG TERM MAINTENANCE IN TISSUE CULTURE. J. Lemann Jr., R.A. Paccillo*, A.G.F. Ruckert*, S.W. Wilson*, R.O. Hussa* and R.W. Gray, Departments of Medicine, Gynecology/Obstetrics, Surgery and Biochemistry, Medical College of Wisconsin, Milwaukee, WI.

Segments of metastatic parathyroid carcinoma from a patient with a serum Ca of 17.2 mg/dl, P 1.6 mg/dl and iPTH 160 uEq/ml (normal 2-10 uEq/ml; Slatopolsky COOH and intact antibody; 1 uEq/ml = about 40 pg bPTH/ml) were explanted into tissue culture 4/23/78 using 50% Waymouth's 752/1, 30% Cey's balanced salt solution and 20% human cord serum as medium. Initial epithelial cell density was estimated at about 6×10^5 cells/flask. Cell proliferation was seen within 4 days and persists. However by 11/79, 1 1/2 years after the cultures were begun epithelial density decreased to about 10^3 to 10^4 cells/flask where it has remained. The initial culture fluid iPTH of 1.5×10^5 uEq/ml or 6 ug bPTH equivalent/ml has declined in parallel to the decrease in cells and has remained at about 100 uEq/ml or 40 ng bPTH/ml, for the past year. Estimated PTH production has remained at about 1 pg/cell/day or 100 attomol/cell/day for 2 1/2 years. Gel filtration of early (5/78) and late (3/80) culture fluids have shown that the peak of iPTH comigrates with 125I-bPTH without smaller fragments of iPTH. Biological activity of early culture fluid PTH was tested in the fetal rat bone system by Dr. Paula Stern, Northwestern University. Bone resorbing activity was simply additive to that of synthetic bPTH 1-34 and was present at a concentration of 6 ug bPTH equivalent/ml, similar to that observed as iPTH. Attempts to stimulate cell proliferation with several growth factors or lowering the medium Ca from 1.2 to 0.8 mM has thus far failed. We conclude that these human malignant parathyroid cells continue to produce intact and biologically active hPTH after 2 1/2 years in tissue culture.

ON THE RELATIONSHIP OF HYPERPARATHYROIDISM AND SERUM GLYCOPROTEINS AND SIALYL TRANSFERASE. A.A. Licata and L. Sheeler*, University of Rochester, Rochester, N.Y. and Cleveland Clinic, Cleveland, Ohio

Although parathyroid hormone (PTH) is metabolized by the liver, it is still unclear whether the hormone affects hepatic function. To study this, we investigated whether serum glycoproteins and sialyl transferase activity (STA), markers of hepatic function, were altered in hyperparathyroidism (HPT). Serum from normal (n=16) and (HPT) (n=20) subjects were analyzed for total precipitability (ppt) and soluble (serum) sialic acid, hexosamine, neutral hexoses, fucose, uronic acid, and protein and for specific glycoproteins by radial immunodiffusion. (STA) was measured by the incorporation of isotopic sialic acid into desialylated fetuin. Initial studies showed that serum proteins (40%) from (HPT) sera were retained on Con-A-agarose than from normal or Pagetic sera and were eluted with methyl-D-mannoside. Total (ppt) carbohydrate content was increased. The increase was greatest for sialic acid and hexosamine ($p < 0.01$). (ppt) hexoses correlated with increased PTH ($r=0.64$) and alkaline phosphatase ($r=0.52$). Serum uronic acid and alkaline phosphatase were unchanged. Specific glycoproteins were either decreased ($p < 0.05$) (transferrin, a-2-macroglobulin) or unchanged (haptoglobin, a-1-antitrypsin). (STA) was similar in (HPT) and normals but correlated ($p < 0.05$) with PTH ($r=0.68$), (ppt) hexoses ($r=0.60$), and alkaline phosphatase ($r=0.60$) in the (HPT). We concluded (1) that (HPT) was associated with increased total carbohydrate content of serum proteins but not necessarily specific glycoproteins. (2) that its disease activity, as noted by changes in PTH and alkaline phosphatase, was reflected by changes in (STA) and (ppt) hexoses, and (3) that there was an unexplained relationship between hepatic glycoprotein metabolism and (HPT).

PROLACTIN-STIMULATED PARATHYROID HORMONE SECRETION IS NOT MEDIATED BY CYCLIC AMP OR CATECHOLAMINES. L. Magliola*, M.L. Thomas and L.R. Forte. Dept. of Pharmacology, Univ. Missouri and Truman VA Hospital, Columbia, Mo.

Recent reports suggest that bovine parathyroid glands contain dopamine (DA), possibly within mast cells, and that DA synthesis and release from rat brain is stimulated by prolactin (PRL). We previously reported that PRL stimulates PTH secretion in dispersed bovine parathyroid cells. The present study was undertaken to explore the possibility that PRL-stimulated PTH release is mediated via endogenous catecholamines. PTH secretion due to PRL was not blocked by either the α -adrenergic or dopaminergic antagonists, propranolol and fluphenazine, respectively. PRL did not increase cyclic AMP above basal levels in parathyroid cells incubated for up to 3 hours with or without 1 mM MIX. DA- and isoproterenol (ISO)-stimulated cyclic AMP were effectively blocked by fluphenazine and propranolol, respectively. These data suggest that PRL-stimulated PTH release is not mediated through the β -adrenergic or dopaminergic receptor systems of parathyroid cells and confirms our previous report that the effect of PRL is not mediated by cyclic AMP. We also found that haloperidol, a DA-antagonist, markedly stimulated PTH release. Haloperidol in combination with maximal levels of DA, or PRL produced additive increases in PTH secretion. Also, haloperidol-stimulated PTH secretion was not blocked by the antagonists, propranolol, phentolamine or atropine. Other DA-antagonists such as fluphenazine and butaclamol did not stimulate secretion. In conclusion, the results of this study suggest that PRL and haloperidol may stimulate PTH secretion through agonist-like activity via receptors other than the β -adrenergic or dopaminergic receptor types.



Differences in Binding Affinities of Human PTH(1–84) Do Not Alter Biological Potency: A Comparison Between Chemically Synthesized Hormone, Natural and Mutant Forms

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OLSTAD, O. K., N. E. MORRISON, R. JEMTLAND, H. JÜPPNER, G. V. SEGRE and K. M. GAUTVIK. *Differences in binding affinities of human PTH(1–84) do not alter biological potency: A comparison between chemically synthesized hormone, natural and mutant forms.* PEPTIDES 15(7) 1261–1265, 1994.—The purpose of this study was to evaluate receptor binding affinities and biological properties in vitro and in vivo of various recombinant hPTH(1–84) forms representing the natural hormone and a mutagenized hPTH form, [Gln²⁶]hPTH(1–84) (QPTH), after expression in *E. coli* and *Saccharomyces cerevisiae*. In LLC-PK₁ cells stably transformed with the rat PTH/PTHrP receptor, chemically synthesized hPTH(1–84) and QPTH showed a reduced binding affinity (apparent K_d 18 and 23 nM, respectively) than the recombinant, hPTH(1–84) (apparent K_d 9.5 nM). All recombinant hPTH forms showed a similar potency to stimulate cellular cAMP production (EC_{50} 1.5 nM) and significantly better than chemically synthesized hPTH (EC_{50} 5.7 nM). All hormone forms showed an about equipotent activity in causing elevation in serum calcium, increased excretion of urine phosphate, and cAMP. Thus, the natural recombinant PTH forms showed higher binding affinities and adenylate cyclase activation potencies in LLC-PK₁ cells, but the reduced receptor binding affinity exerted by QPTH did not transcend differences in cAMP generation and in vivo biological activities.

Recombinant parathyroid hormones Recombinant PTH/PTHrP receptor cAMP response Rats

PARATHYROID hormone is the principle regulator of calcium homeostasis in humans and has been advanced as an anabolic drug against postmenopausal osteoporosis (22,25). The hormone, which is produced in the mammalian parathyroid glands, is synthesized as an 115 amino acid precursor that is processed to the mature hormone of 84 amino acids (21). The information required for high-affinity binding of PTH to its receptor in bone and kidney cells is contained within the biologically active 1–34 region (20). The amino-terminus of PTH is essential for triggering the adenylate cyclase response pathway (8,26), but it also contributes modestly to receptor binding affinity. In addition to a nearly complete loss of cAMP agonism, the deletion of residues 1–6 is accompanied by an approximately 100-fold decrease in receptor binding affinity (7,10,18,24). The major component of PTH receptor binding affinity, however, appears to be determined by residues 28–34. Deletion of these residues causes at least a 1000-fold reduction in binding affinity (18). Furthermore,

PTH(25–34) displays weak, but detectable, receptor binding affinity ($K_d \approx 100 \mu M$) (18). In comparison, no evidence for receptor interaction has been obtained for amino-terminal fragments shorter than PTH(1–27) (24,26). Based on these observations, the 25–34 region has been called the hormone's principal receptor binding domain (18).

We have previously reported production of hPTH(1–84) in yeast (5), and the α -factor expression system is a well-characterized, commonly used strategy for expression of foreign proteins by the yeast *Saccharomyces cerevisiae* (3,27,29). The mating factor alpha (MF α) leader sequence is cleaved off sequentially by the KEX-2 endopeptidase and then by an amino peptidase STE13, leaving a correct N-terminal after guiding the recombinant protein through the secretory pathway (11). In the expression plasmid p α UXPTH-2, the MF α promoter, signal sequence, and termination signal were employed. The secreted hormone was purified from medium to more than 95% homo-

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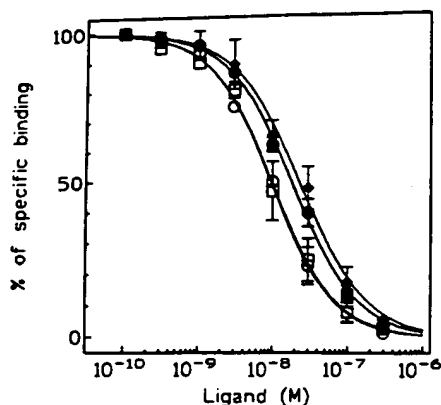


FIG. 1. Inhibition of radiolabeled [Tyr³⁶]chickenPTHrP(1-36)amide by different hPTHs. Recombinant hPTH(1-84) produced in *E. coli* (○), recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* (□), [Gln²⁶]hPTH(1-84) (QPTH) produced in *Saccharomyces cerevisiae* (◆), and chemically synthesized hPTH(1-84) (●) were tested in radioreceptor assay using LLC-PK₁ cells transfected with the rat PTH/PTHrP receptor. The data represent the mean \pm SD of at least two independent experiments, each performed in triplicate.

geneity, characterized chemically, and shown to represent the natural hormone (5,19). In addition to the intact hormone, an aberrant KEX-2 cleavage occurring at an internal site (5) after two consecutive basic amino acids in the hPTH sequence -Arg²⁵-Lys²⁶↓Lys²⁷-resulted in part fragmentation of the hormone. To improve the yield of hPTH, and to avoid internal degradation, a point mutation was introduced into the gene, changing Lys in position 26 to Gln (Q) (23). The resulting agonist, [Gln²⁶]hPTH(1-84), called QPTH, was tested together with recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* and compared with chemically synthesized hPTH(1-84) in certain biochemical and biological tests.

We have also produced full-length hPTH in *E. coli* as a secretory product employing the *Staphylococcus aureus* protein A signal and regulatory sequences (9). After purification from

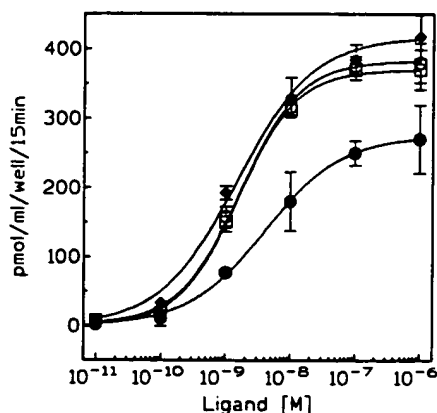


FIG. 2. Stimulation of cAMP by different hPTHs. Accumulation of intracellular cAMP in LLC-PK₁ cells transfected with the rat PTH/PTHrP receptor stimulated (15 min, 37°C) with recombinant hPTH(1-84) produced in *E. coli* (○), recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* (□), [Gln²⁶]hPTH(1-84) (QPTH) produced in *Saccharomyces cerevisiae* (◆), and chemically synthesized hPTH(1-84) (●). The data represent the mean \pm SD of two independent experiments, each performed in duplicate.

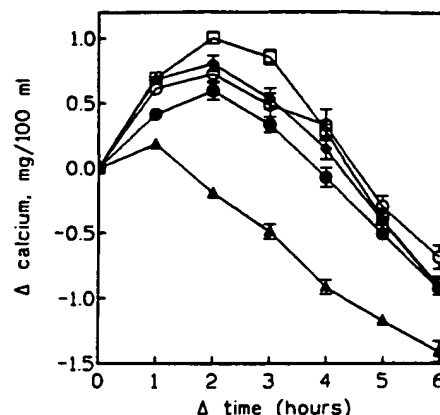


FIG. 3. Induction of hypercalcemia by different hPTHs. Parathyroidectomized male Wistar rats were administered different forms of hPTH (2.0 μ g of the recombinant hPTHs, 2.7 μ g of the chemically synthesized hPTH) as described in the Method section. The stimulating agents were recombinant hPTH(1-84) produced in *E. coli* (○), recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* (□), [Gln²⁶]hPTH(1-84) (QPTH) produced in *Saccharomyces cerevisiae* (◆), and chemically synthesized hPTH(1-84) (●). Control (▲). Blood samples were drawn at 0, 1, 2, 3, 4, 5, and 6 h after injection of PTH. The results are reported as the difference between the amount of calcium in the blood at the various time points, subtracting out the amount of calcium in the baseline sample (delta values). The data represents the mean \pm SEM ($n = 6$).

medium and chemical characterization, this recombinant form was also included in the biochemical and biological characterizations.

LLC-PK₁ cells (porcine renal epithelial cells) stably transfected with the cDNA for the rat PTH/PTHrP receptor (4) were used for the receptor binding studies and cAMP responsiveness; rats

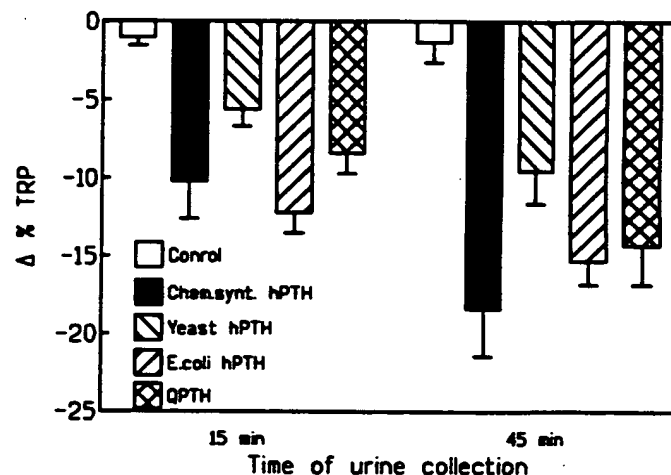


FIG. 4. Urinary excretion of phosphate. Parathyroidectomized male Wistar rats were administered different forms of PTH (2.0 μ g of the recombinant hPTHs, 2.7 μ g of the chemically synthesized hPTH) as described in the Method section. Urine was collected for two periods: 0-30 and 30-60 min after administration of PTH. The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP) and is calculated by the formula: $(1 - \text{phosphate clearance} / \text{creatinine clearance}) \times 100$. The result is reported as a change in % TRP related to the zero control level, and a decrease represents a greater amount of phosphate excreted in the urine. The data represents the mean \pm SEM ($n = 6$).

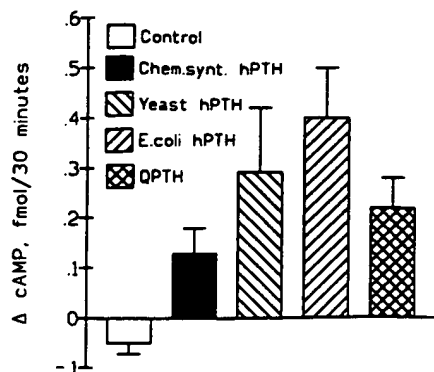


FIG. 5. Changes in urinary cAMP after administration of PTH. Parathyroidectomized male Wistar rats were administered different forms of PTH (2.0 μ g of the recombinant hPTHs, 2.7 μ g of chemically synthesized hPTH) as described in the Method section. Urine was collected for 30 min after administration of PTH. The excretion of cAMP is reported as a change in cAMP concentration related to the zero control level. The data represents the mean \pm SEM ($n = 6$).

were used for measurements of the hypercalcemic response, urine phosphate, and cAMP.

METHOD

Chemically synthesized hPTH(1-84) was purchased from Bachem Fine Chemicals (Torrance, CA) and [Tyr³⁶]chicken-PTHrP(1-36)-NH₂ for radioiodination was from Peninsula Laboratories. The production, purification, and chemical characterization of recombinant PTHs have been described previously (5,9,19,23). Peptide concentrations were determined by amino acid analysis. The blood and urine samples were analyzed for calcium, phosphate, protein, and creatinine on the Cobas Bio Autoanalyzer. cAMP was analyzed using a commercial radioimmunoassay kit from Amersham. All reagents were of highest purity available.

Radioreceptor Assay

LLC-PK₁ cells expressing the rat PTH/PTHrP receptor (4) were plated in 24-well plates. The cells were incubated with [¹²⁵I]-labeled [Tyr³⁶]chickenPTHrP(1-36)-NH₂ (100,000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 h, using a Tris-based binding buffer (50 mM Tris-HCl), pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal calf serum as described (28). The competing ligands included chemically synthesized hPTH(1-84) from Bachem, recombinant hPTH(1-84) expressed in *E. coli* (9), recombinant hPTH(1-84) expressed in yeast (5,19), and recombinant QPTH expressed in yeast (23). Techniques used for radioiodination of PTHrP analogue have been reported (12,13). PTH and PTHrP bind to and activate PTH receptors in bone and kidney in an indistinguishable manner (12,14). [¹²⁵I][Tyr³⁶]chickenPTHrP(1-36)-NH₂ was used as radioligand because of lower nonspecific binding (less than 5% of total binding) (14) compared to [¹²⁵I][Nle^{8,18}Tyr³⁴]bovine-PTH(1-34)-NH₂, which gave 10–15% nonspecific binding (28).

Intracellular cAMP Measurements

For measurements of intracellular cAMP, LLC-PK₁ cells (4) expressing the rat PTH/PTHrP receptor were plated in 24-well plates (50,000 cells/well) and grown to confluence for 3 days

(about 250,000 cells/well). The cells were placed on ice, rinsed once with 1 ml of cold Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, and 0.1% bovine serum albumin. Medium (0.5 ml) with or without PTH was added and cells were transferred to a 37°C water bath for incubation in 15 min. Then the cells were rinsed once with 0.5 ml phosphate-buffered saline and immediately frozen on liquid nitrogen. Intracellular cAMP was measured by a radioimmunoassay kit from Amersham, after lysing the cells with 1 ml of 0.05 N HCl.

Hypercalcemic Assay

Male Wistar rats (150–200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. Preliminary experiments showed that this was a reliable way to obtain complete removal of parathyroid gland activity because plasma calcium fell linearly as a function of time, as also indicated by the control group in Fig. 3. Moreover, individual rats showed small variations in the results. The parathyroid glands were removed for two reasons. One, to eliminate the endogenous production of the hormone, and two, to make the animals more sensitive to exogenous hormone. The increase in sensitivity is assumed to be due to the upregulation of PTH receptors in target organs (16). Thus, it has previously been shown that tubular membranes prepared from parathyroidectomized rats reveal a higher binding of [³H]hPTH(1-34) and higher maximum stimulation of PTH-stimulated adenylate cyclase compared to control animals (16). It has also been shown (28) that downregulation of PTH receptors in ROS 17/2 cells occurs when the cells are exposed to PTH concentrations near hormonal physiological doses. More than 50% of the of the PTH-stimulated adenylate cyclase activity was recovered within 24 h after desensitization.

The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml/rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate, 4 % bovine serum albumin, 25 units heparin/ml. Five minutes after injection of 200 μ l of the heparinized Ringers acetate, a baseline blood sample was drawn (300 μ l). The animals were tracheotomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. The PTH agonist was then injected SC in a volume of 200 μ l. All agonists were dissolved into 100 μ l of 0.01 N acetic acid. The test agents included:

1. vehicle, 0.001 N acetic acid, 1% bovine serum albumin (control),
2. chemically synthesized human PTH(1-84), 2.7 μ g/rat (chem. synt. hPTH),
3. recombinant human PTH(1-84) from yeast, 2.0 μ g/rat (yeast hPTH),
4. recombinant human PTH(1-84) from *E. coli*, 2.0 μ g/rat (*E. coli* hPTH),
5. recombinant [Gln²⁶]hPTH(1-84) from yeast, 2.0 μ g/rat (QPTH).

Due to the reduced receptor binding affinity and cAMP stimulation in the in vitro assays below, the chemically synthesized hPTH concentration was used at 2.7 μ g/rat.

After dissolving in acetic acid, the agents were brought up in 900 μ l of Ringers acetate containing 1% bovine serum albumin. Blood samples were drawn at 1, 2, 3, 4, 5, and 6 h after injection of hPTH or agonist. The rats were reheparinized 5 min before

drawing each blood sample using 200 μ l of the heparinized Ringers solution.

All forms of hPTH were analyzed and quantified by amino acid analysis before administration to the rats.

The blood samples were centrifuged in a clinical centrifuge for 10 min, then the plasma was analyzed for calcium using a Cobas Autoanalyzer.

Urine Analysis

Male Wistar rats (150–200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml/rat). The carotid artery and the jugular vein were cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate, 4% bovine serum albumin, 25 units heparin/ml. The bladder was catheterized using PE-200 tubing.

The carotid artery was cannulated for the collection of blood samples, and the jugular vein was cannulated for the purpose of injecting the hormones, and for a slow infusion for the purpose of volume loading the rats to increase the urine output. The rats were infused with Ringers acetate, 4% bovine serum albumin at the rate of 3 ml/h. The infusion was run for 2 h before the start of the experiment to equilibrate the animals.

After the 2-h equilibration period, a baseline urine collection was made for 30 min, with a midpoint arterial blood sample drawn at 15 min. At the end of the baseline urine collection, the PTH was injected IV, and a new 30-min urine collection was started. Again, a midpoint blood sample was taken 15 min into the urine collection. A final 30-min urine collection was made from 30–60 min after the injection of PTH, with the midpoint blood collection made at 45 min after PTH injection.

The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP). The % TRP is calculated by the formula: $(1 - \text{phosphate clearance/creatinine clearance}) \times 100$. A decrease of % TRP represents a greater amount of phosphate excreted in the urine. The creatinine clearance did not change in any of the treatment groups. It was not expected to change, and was only measured to calculate the % TRP.

Statistical Analyses

A two-sample *t*-test was used comparing mean values of control and treated groups of animals (2).

RESULTS

Radioreceptor Binding Studies and Intracellular cAMP Measurements

Binding of the different hPTH forms is shown in terms of displacement curves using the [125 I][Tyr 36]chicken PTHrP(1–36)-NH $_2$ as radioligand and LLC-PK $_1$ cells permanently transfected with the rat PTH/PTHrP receptor.

The chemically synthesized hPTH and QPTH had calculated binding affinities with K_d of 18 nM (95% confidence interval: 16.1–20.0 nM) and 23 nM (95% confidence interval: 19.0–27.2 nM), respectively (Fig. 1). The natural recombinant hPTH(1–84) forms from *Saccharomyces cerevisiae* and *E. coli* had a similar but significantly lower apparent K_d of 9.5 nM (95% confidence interval: 8.7–10.4 nM) (Fig. 1). In spite of these differences in receptor binding affinities, all the recombinant hormones had equal ability to stimulate intracellular cAMP accumulation (EC $_{50}$ about 1.5 nM, 95% confidence interval: 1.0–2.2 nM) (Fig. 2). In contrast, the synthetic hPTH showed a significant reduced potency to stimulate cAMP production with EC $_{50}$ of 5.7 nM

(95% confidence interval: 3.4–9.6 nM) on a molar basis, and a reduced maximal response.

Hypercalcemic Assay

After parathyroidectomy, the control calcium concentration fell linearly 1 h after the operation (about 0.75 mg %/h) (Fig. 3). The chemically synthesized hPTH was injected in a dose of 2.7 μ g/rat compared to 2.0 μ g/rat employed for the other hPTH species due to the reduced receptor binding affinity and cAMP stimulation of the chemically synthesized hPTH. These concentrations were chosen on the basis of preliminary experiments using a range of different doses and were selected because they gave a healthy hypercalcemic response and no observable side effects (e.g., unaffected rectal temperature). The hypercalcemic response of the chemically synthesized preparation was somewhat lower than for yeast hPTH, but almost similar to the other recombinant hPTHs. From these experiments it appeared that the declining parts of the curves were similar and like the slope of the control curve (Fig. 3).

Tubular Reabsorption of Phosphate

The percent tubular reabsorption of phosphate (% TRP) was calculated on basis of urine creatinine values and showed a strong and significant ($p < 0.01$) reduction after injection of the different PTH forms, and the potencies were similar. This effect was already observed 15 min after injection, and was then close to or at its maximum (Fig. 4). (For calculation of % TRP, the Method section.)

Measurements of Cyclic Adenosine Monophosphate

The changes in the cAMP content of the urine after administration of PTH was somewhat variable, with the chemically synthesized hPTH showing the smallest effect. However, all forms of PTH responded in a similar fashion. Therefore, there is no principal difference between the preparations of PTH in terms of their stimulation of cAMP release into the urine (Fig. 5).

DISCUSSION

Structural analysis of PTH indicates that PTH(19–34) fragment contains substantial helical structure (17) and the residues 17–28 form an α -helix (15). This assumption has been confirmed (6), showing that mutations of the hydrophobic residues Leu 24 , Leu 28 , and Val 31 in hPTH are critical for optimal PTH activity, in contrast to most mutations of the polar residues (i.e., Lys 26 , Lys 27 , Gln 29 , Asp 30 , and His 32).

We have previously showed that QPTH is fully active in assays of adenylate cyclase, and this observation has been confirmed (6). Also in bone resorption studies using mouse calvaria (23), the QPTH was equally potent compared with the natural hormone. Biotinylation of Lys 26 or Lys 27 of [Nle 8,18 , Tyr 34]bPTH(1–34) has no effect on binding affinity (1), but substitutions as Lys $^{26} \rightarrow$ Glu and Lys $^{26} \rightarrow$ Thr causes partial reduction in cAMP production by PTH stimulation (6).

Interestingly, the substitution in QPTH, Lys $^{26} \rightarrow$ Gln, lowers the hormone's affinity to the receptor 2.4 times, but does not influence the cAMP production compared to the wild-type hormone, indicating that the efficacy of the hormone receptor complex to stimulate the cyclase dependent G-protein(s) may still be similar. This certainly also shows that it is important to complement receptor binding studies with functional analysis.

Our in vivo studies have shown that the recombinant forms of hPTH are at least as potent as chemically synthesized hPTH

(29% higher doses of the chemically synthesized preparation were used), demonstrating that the yeast and *E. coli* hPTHs were correctly processed and that the molecule folded correctly to give the proper tertiary structure, which is necessary to give full biological activity. Also, QPTH has folding characteristics that make it as active as the natural hormone. However, the reduced receptor binding potency and biological responses of the chemically synthesized hPTH on a molar basis is unexplained, but may be related to an inadequate N-terminal structure that is the

last synthesized part of the peptide and for PTH is of crucial importance for receptor binding and eliciting the biological responses.

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REFERENCES

1. Abou-Samra, A. B.; Freeman, M.; Juppner, H.; Uneno, S.; Segre, G. V. Characterization of fully active biotinylated parathyroid hormone analogs. Application to fluorescence-activated cell sorting of parathyroid hormone receptor bearing cells. *J. Biol. Chem.* 265:58-62; 1990.
2. Altman, D. G., ed. *Practical statistics for medical research*. London: Chapman & Hall; 1991:189-198.
3. Brake, A. J.; Merryweather, J. P.; Coit, D. G.; et al. Alpha-factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 81:4642-4646; 1984.
4. Bringhurst, F. R.; Juppner, H.; Guo, J.; et al. Cloned, stably expressed parathyroid hormone (PTH)/PTH-related peptide receptors activate multiple messenger signals and biological responses in LLC-PK1 kidney cells. *Endocrinology* 132:2090-2098; 1993.
5. Gabrielsen, O. S.; Reppe, S.; Saether, O.; et al. Efficient secretion of human parathyroid hormone by *Saccharomyces cerevisiae*. *Gene* 90:255-262; 1990.
6. Gardella, T. J.; Wilson, A. K.; Keutmann, H. T.; et al. Analysis of parathyroid hormone's principal receptor-binding region by site-directed mutagenesis and analog design. *Endocrinology* 132:2024-2030; 1993.
7. Goldman, M. E.; Chorev, M.; Reagan, J. E.; Nutt, R. F.; Levy, J. J.; Rosenblatt, M. Evaluation of novel parathyroid hormone analogs using a bovine renal membrane receptor binding assay. *Endocrinology* 123:1468-1475; 1988.
8. Goltzman, D.; Peytremann, A.; Callahan, E.; Tregear, G. W.; Potts, J. T., Jr. Analysis of the requirements for parathyroid hormone action in renal membranes with the use of inhibiting analogues. *J. Biol. Chem.* 250:3199-3203; 1975.
9. Hogset, A.; Blingsmo, O. R.; Saether, O.; et al. Expression and characterization of a recombinant human parathyroid hormone secreted by *Escherichia coli* employing the staphylococcal protein A promoter and signal sequence. *J. Biol. Chem.* 265:7338-7344; 1990.
10. Horiuchi, N.; Holick, M. F.; Potts, J. T., Jr.; Rosenblatt, M. A parathyroid hormone inhibitor in vivo: Design and biological evaluation of a hormone analog. *Science* 220:1053-1055; 1983.
11. Julius, D.; Blair, L.; Brake, A.; Sprague, G.; Thorne, J. Yeast alpha factor is processed from a larger precursor polypeptide: The essential role of a membrane-bound dipeptidyl aminopeptidase. *Cell* 32:839-852; 1983.
12. Juppner, H.; Abou-Samra, A. B.; Uneno, S.; Gu, W. X.; Potts, J. T., Jr.; Segre, G. V. The parathyroid hormone-like peptide associated with humoral hypercalcemia of malignancy and parathyroid hormone bind to the same receptor on the plasma membrane of ROS 17/2.8 cells. *J. Biol. Chem.* 263:8557-8560; 1988.
13. Juppner, H.; Abou-Samra, A. B.; Uneno, S.; Keutmann, H. T.; Potts, J. T., Jr.; Segre, G. V. Preparation and characterization of [N alpha-(4-azido-2-nitrophenyl)Ala1,Tyr36]-parathyroid hormone related peptide (1-36)amide: A high-affinity, partial agonist having high cross-linking efficiency with its receptor on ROS 17/2.8 cells. *Biochemistry* 29:6941-6946; 1990.
14. Kaufmann, M.; Fischer, J. A.; Muff, R. Comparison of parathyroid hormone receptors in rat osteosarcoma cells and kidney. *Biochim. Biophys. Acta* 1179:197-202; 1993.
15. Klaus, W.; Dieckmann, T.; Wray, V.; Schomburg, D.; Wingender, E.; Mayer, H. Investigation of the solution structure of the human parathyroid hormone fragment (1-34) by 1H NMR spectroscopy, distance geometry, and molecular dynamics calculations. *Biochemistry* 30:6936-6942; 1991.
16. Loreau, N.; Lajotte, C.; Lafaye, M.; Ardaillou, R. Renal receptors for parathyroid hormone in normal, parathyroidectomized and vitamin D-deficient rats. In: Massry, S. G.; Ritz, E.; Jahn, H., eds. *Phosphate and minerals in health and disease*. New York: Plenum Press; 1980:537-545.
17. Neugebauer, W.; Surewicz, W. K.; Gordon, H. L.; Somorjai, R. L.; Sung, W.; Willick, G. E. Structural elements of human parathyroid hormone and their possible relation to biological activities. *Biochemistry* 31:2056-2063; 1992.
18. Nussbaum, S. R.; Rosenblatt, M.; Potts, J. T., Jr. Parathyroid hormone-renal receptor interactions. Demonstration of two receptor-binding domains. *J. Biol. Chem.* 255:10183-10187; 1980.
19. Olstad, O. K.; Reppe, S.; Gabrielsen, O. S.; et al. Isolation and characterization of two biologically active O-glycosylated forms of human parathyroid hormone produced in *Saccharomyces cerevisiae*. Identification of a new motif for O-glycosylation. *Eur. J. Biochem.* 205:311-319; 1992.
20. Potts, J. T., Jr.; Tregear, G. W.; Keutmann, H. T.; et al. Synthesis of a biologically active N-terminal tetratriacontapeptide of parathyroid hormone. *Proc. Natl. Acad. Sci. USA* 68:63-67; 1971.
21. Potts, J. T., Jr.; Kronenberg, H. M.; Habener, J. F.; Rich, A. Biosynthesis of parathyroid hormone. *Ann. NY Acad. Sci.* 343:38-55; 1980.
22. Reeve, J.; Meunier, P. J.; Parsons, J. A.; et al. Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: A multicentre trial. *Br. Med. J.* 280:1340-1344; 1980.
23. Reppe, S.; Gabrielsen, O. S.; Olstad, O. K.; et al. Characterization of a K26Q site-directed mutant of human parathyroid hormone expressed in yeast. *J. Biol. Chem.* 266:14198-14201; 1991.
24. Rosenblatt, M.; Segre, G. V.; Tyler, G. A.; Shepard, G. L.; Nussbaum, S. R.; Potts, J. T., Jr. Identification of a receptor-binding region in parathyroid hormone. *Endocrinology* 107:545-550; 1980.
25. Slovik, D. M.; Rosenthal, D. I.; Doppelt, S. H.; et al. Restoration of spinal bone in osteoporotic men by treatment with human parathyroid hormone (1-34) and 1,25-dihydroxyvitamin D. *J. Bone Miner. Res.* 1:377-381; 1986.
26. Tregear, G. W.; Van Rietschoten, J.; Greene, E.; et al. Bovine parathyroid hormone: Minimum chain length of synthetic peptide required for biological activity. *Endocrinology* 93:1349-1353; 1973.
27. Vlasuk, G. P.; Bencen, G. H.; Scarborough, R. M.; et al. Expression and secretion of biologically active human atrial natriuretic peptide in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 261:4789-4796; 1986.
28. Yamamoto, I.; Shigeno, C.; Potts, J. T., Jr.; Segre, G. V. Characterization and agonist-induced down-regulation of parathyroid hormone receptors in clonal rat osteosarcoma cells. *Endocrinology* 122:1208-1217; 1988.
29. Zsebo, K. M.; Lu, H. S.; Fieschko, J. C.; et al. Protein secretion from *Saccharomyces cerevisiae* directed by the prepro-alpha-factor leader region. *J. Biol. Chem.* 261:5858-5865; 1986.

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The mucosal immune system: from fundamental concepts to vaccine development

Jerry R. McGhee^{*§}, Jiri Mestecky^{*}, Mark T. Dertzbaugh[†], John H. Eldridge[‡], Masatomo Hirasawa[†] and Hiroshi Kiyono[†]

Recent studies in experimental animals and humans have shown that the mucosal immune system, which is characterized by secretory IgA (S-IgA) antibodies as the major humoral defence factor, contains specialized lymphoid tissues where antigens are encountered from the environment, are taken up and induce B- and T-cell responses. This event is followed by an exodus of specific lymphocytes, which home to various effector sites such as the lamina propria regions and glands. These responses are regulated by T cells and cytokines and lead to plasma cell differentiation and subsequent production of S-IgA antibodies in external secretions. This knowledge has led to practical approaches for vaccine construction and delivery into mucosal inductive sites in an effort to elicit host protection at mucosal surfaces where the infection actually occurs.

Keywords: Mucosal; secretory IgA; lamina propria; Peyer's patches; lymphocytes

THE MUCOSAL IMMUNE SYSTEM

Among the most important protective humoral immune factors at mucosal surfaces where most pathogens enter the host are locally produced antibodies of the secretory IgA (S-IgA) isotype. This isotype constitutes >80% of all antibodies produced in mucosa-associated tissues (reviewed in Refs 1 and 2), and S-IgA is induced, transported and regulated by mechanisms that are remarkably distinct from those involved in systemic antibody responses¹⁻³. The mucosal immune system can be divided into sites where antigen is encountered and initial responses are induced and into the larger surface areas where IgA plasma cells are found and where the production of S-IgA antibodies results in local immune protection. It is now well established that environmental antigens, which are most often encountered by inhalation or ingestion, can be taken up into specialized lymphoreticular tissues in the upper respiratory tract (URT) and the gastrointestinal (GI) tract^{4,5}. These are termed, respectively, bronchus-associated and gut-associated

lymphoid tissues (BALT and GALT)¹⁻³. A number of studies in several experimental animal models including mice and in humans have provided compelling evidence that the stimulation of IgA precursor B cells in GALT with orally administered antigens leads to the dissemination of B and T cells to mucosal effector tissues such as lamina propria regions of the intestinal, respiratory and genitourinary tracts and various secretory glands for subsequent antigen-specific S-IgA antibody responses^{1-3,6-10}. Further, several oral vaccines have been shown to induce appropriate S-IgA responses in remote secretions including saliva, tears and fluids obtained from nasal and gastrointestinal washes (reviewed in Refs 1 and 10). Thus, it is feasible to consider that most vaccines which are currently given by the systemic route and those currently under development could be considered for oral administration. Oral immunization is both practical and safe in terms of lack of side effects and can induce protective mucosal S-IgA responses. However, oral administration of proteins, including subunit vaccines, may induce a state of unresponsiveness, termed oral tolerance¹¹.

Therefore, in order to achieve the goal of effective oral immunization, we must define antigen delivery systems better and simultaneously measure both humoral and cellular responses to the vaccine epitopes. This will require studies of regulatory cells including T cells as well as effector lymphocytes (both B and T cells) in mucosal responses. Finally, the end result of oral immunization, e.g. the induction of protective immunity, must be unequivocally assessed at the site of mucosal entry by the pathogen.

IgA inductive sites

Most studies of IgA inductive sites have been performed on GALT, which is collectively represented by the Peyer's patches (PP), the appendix, and small solitary lymphoid nodules. The BALT shares many anatomical similarities with GALT, and probably serves the same functions in the URT^{5,12}. The PP contain a dome region enriched for lymphocytes, macrophages and some plasma cells. This dome area is covered by a unique epithelium enriched for specialized antigen-sampling cells, termed follicle-associated epithelial (FAE) or microfold (M) cells, which exhibit thin extensions around

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lymphoid cells¹³⁻¹⁵. The FAE or M cells have short microvilli, small cytoplasmic vesicles and few lysosomes, are adept at uptake and transport of luminal antigens, including proteins and particulates such as viruses, bacteria and even small parasites¹⁵. Antigen uptake by M cells does not result in degradation, but instead of delivery of intact antigen into the underlying lymphoid tissue¹⁵.

Distinct follicles (B cell zones) occur beneath the dome region of PP. The follicles contain germinal centres, where significant B cell division is seen (Table 1). These germinal centres are considered to be sites where frequent B cell switches to IgA and affinity maturation occur and these germinal centres contain the majority of surface IgA⁺ (sIgA⁺) B cells^{16,17}. However, unlike immune lymph nodes and spleen, the B cell zones do not give rise to significant numbers of plasma cells. Adjacent to follicles are the T-cell dependent areas, which contain all major T cell subsets (Table 1). The T cells present in the PP are mature, e.g. they contain a T-cell receptor CD3 complex, and >95% of these T cells use the α/β form of T cell receptor (TCR). Approximately 60% of PP T cells are CD3⁺, CD4⁺, CD8⁻ and exhibit properties of T-helper cells including support for IgA responses³. Nevertheless, significant numbers of CD3⁺, CD4⁻, CD8⁺ T cells are also present in PP, and one can induce functional cytotoxic T lymphocytes (CTLs) with this phenotype in the PP^{3,18}.

Thus, all necessary immunocompetent cells, including CD4⁺ Th cells, CD8⁺ CTLs, sIgA⁺ B cells and accessory cells (class II⁺ B cells, -dendritic cells and -macrophages) are present in IgA inductive sites (Table 1). These cells are engaged in regulation of induction of antigen-specific effector cells which will ultimately mediate both humoral and cellular immune responses for mucosal protection. Thus, by use of appropriate oral antigen delivery, one can now study the induction of CD4⁺ Th cells and sIgA⁺ B cells to protein epitopes. For virus vaccines, it will be important to determine if antigen-specific, CD8⁺ CTLs, which exhibit cytolytic functions, are also optimally induced in the mouse PP.

Mucosal effector sites

Following antigen stimulation in PP and its presentation to B and T cells, the antigen-induced B and T cells leave the PP via efferent lymphatics and reach the

systemic circulation through the thoracic duct. These lymphocytes then enter the effector sites such as the lamina propria of the respiratory, GI and reproductive tracts, and glandular tissues, where these lymphoid cells are selectively retained by mechanisms not yet understood. The B cells clonally expand under the influence of antigen, T cells and cytokines, and become mature IgA plasma cells^{1,2}.

The lamina propria of the GI tract is a major mucosal effector site and the main cell types found are lymphocytes, including 20–40% B cells (including plasma cells) and 40–60% T cells (Table 1). Other important cell types include macrophages ($\approx 10\%$), eosinophils ($\approx 5\%$) and mucosal mast cells ($\approx 1-3\%$). Although large numbers of plasma cells, mostly of the IgA isotype are seen, T cells, as indicated, are the most frequently isolated cell type seen in these regions¹⁹⁻²¹. The majority of T cells are CD3⁺, CD4⁺, CD8⁻ and exhibit helper functions²⁰. Approximately one third of the T cells in these effector regions are CD3⁺, CD4⁻, CD8⁺, and may exhibit CTL (or possibly suppressor) functions (Table 1). More studies are required to determine the precise functions of these two major effector T cell subsets in orally immunized as well as in normal animals. Macrophages found in significant numbers in the lamina propria²¹ may be involved in antigen processing and presentation at this site. Only limited information is available at present on antigen-specific CD4⁺ Th cell and sIgA⁺ B cell interactions in effector tissues. It is important to understand the cellular and molecular interactions between these cells for induction of local responses to vaccine antigens. Further, the induction of functional CD8⁺ CTLs in effector sites must also be studied in more detail.

Ly-1⁺ B cells as precursors of IgA plasma cells

Early experiments in rabbits⁴ have convincingly demonstrated that the GALT, represented primarily by PP, serves as a source of precursors of IgA plasma cells that populate distant tissues including the lamina propria of the intestines. Numerous other studies have now shown that PP B cell repopulation of the mammary, lacrimal, salivary, and uterine glands, the respiratory tract, and, to a lesser degree, spleen and lymph nodes also occur¹. However, it has recently been shown that significant numbers of IgA plasma cells in the murine GI tract can be derived from a self-renewing Ly-1⁺ (CD5⁺)

Table 1 Unique characteristics of immune cells in IgA inductive and effector tissues

Tissues	Cell subsets	Characteristics
Peyer's patch (PP)	CD3 ⁺ , CD4 ⁺ , CD8 ⁻ T cell	Support preferentially IgA responses. Equal frequency of Th1 and Th2 type cells. Approximately 65% of CD3 ⁺ T cells
	CD3 ⁺ , CD4 ⁻ , CD8 ⁺ T cell	Precursor cells for cytotoxic T lymphocytes. Inhibition of immune responses. Approximately 25% of CD3 ⁺ T cells
	CD3 ⁺ , CD4 ⁻ , CD8 ⁻ T cell	Conversion of oral tolerance. γ/δ TCR bearing cells. Approximately 5% of CD3 ⁺ T cells
	sIgA ⁺ B cell	High frequency of IgA committed B cells (5–10% of B cells). Presence of memory and virgin (germinal centre) cells
	Accessory cells (MØ and DC)	Effective antigen-presenting cells. DC-T cell cluster support IgA synthesis. Less than 5% of PP cells
Lamina propria of intestine	CD3 ⁺ , CD4 ⁺ , CD8 ⁻ T cell	Support IgA responses. Higher frequency of Th2 type cells. Approximately 50% of CD3 ⁺ T cells
	CD3 ⁺ , CD4 ⁻ , CD8 ⁺ T cell	Down regulation of IgA synthesis? Effector CTLs. Approximately 30% of CD3 ⁺ T cells
	CD3 ⁺ , CD4 ⁻ , CD8 ⁻ T cell	Unknown (protection of Th cells?) Approximately 10% of CD3 ⁺ T cells
	IgA plasma cells	Approximately 20% of lamina propria cells

Oral Antigen Delivery

CT or CT-B Conjugates
Microcapsules
Live vectors
(Poliovirus
Adenovirus
Salmonella
BCG)

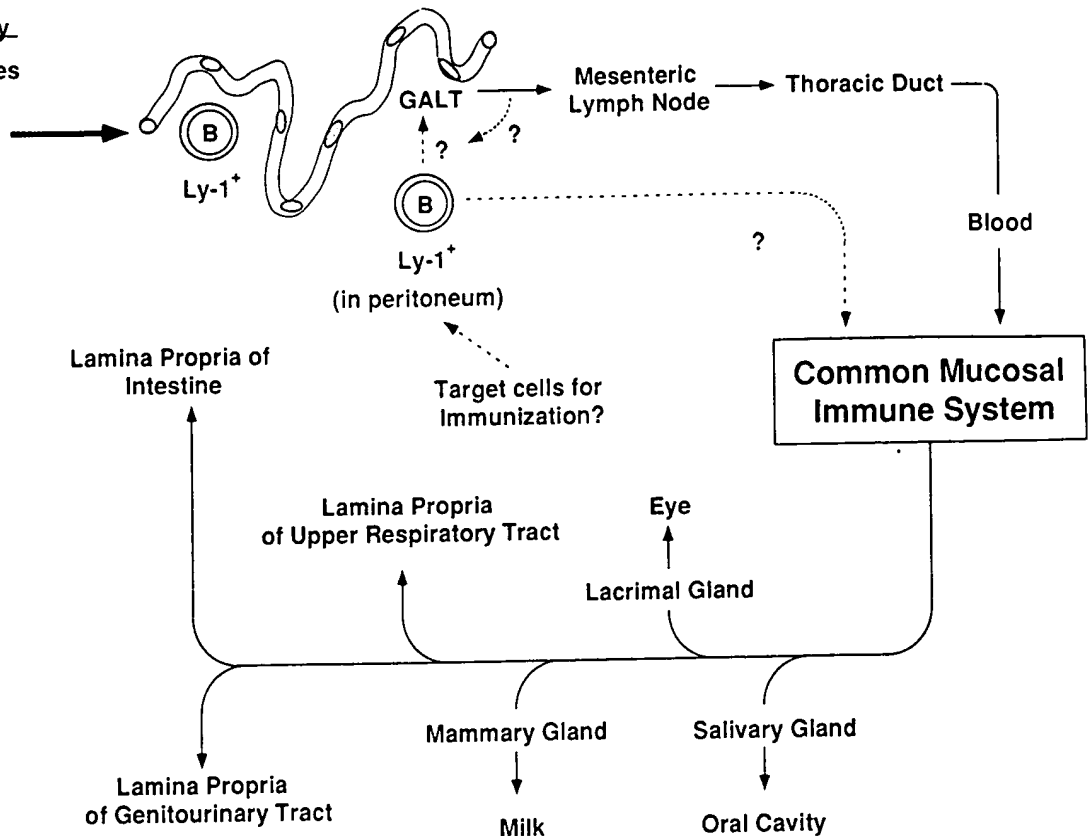


Figure 1 Induction of antigen-specific mucosal immune responses by the common mucosal immune system. Possible antigen delivery systems for the priming of IgA-committed B cells in IgA inductive sites (e.g. GALT) and alternative target B cells (Ly-1⁺ B cells) for the induction of IgA responses

B cell population which resides in the peritoneal cavity²². Recent studies have suggested that the Ly-1⁺ B cell lineage may differ from B cells which arise in the bone marrow²², since they are derived from fetal omentum²³ and exhibit unique phenotypic and localization properties^{22,24}. It is now established that these Ly-1⁺ B cells give rise to serum IgM, with specificity for naturally encountered antigens, as well as to a variety of autoantibodies^{25,26}. It is not yet established if the IgA produced by plasma cells derived from the peritoneal Ly-1⁺ B cell subset also exhibits specificities for natural and self antigens. It is also not known if B cells from PP migrate to the peritoneum and form part of this Ly-1⁺ lineage. However, it has been shown that the PP are markedly deficient in Ly-1⁺ B cells²⁵. It is possible that the initial encounters with antigen in the gut induce antigen-specific B cell responses and that subsequent memory B cells express Ly-1⁺ and take up residence in the peritoneum (Figure 1). Alternatively, the PP and peritoneal B cell subsets may represent non-overlapping populations. Memory B cells in this site could represent a continued source of antigen-specific B cells for mucosal tissues. Thus, both PP and the peritoneum may represent sites for development of B cells capable of repopulating mucosal tissues. In this regard, in order to induce maximum antigen-specific IgA responses at the mucosal surface area, one must consider the possibility that Ly-1⁺ B cells can be target cells for immunization to induce antigen-specific IgA responses (Figure 1).

The common mucosal immune system and oral vaccines

Following antigen presentation by PP accessory cells, B and T cells leave the PP in efferent lymphatics and reach the systemic circulation through the thoracic duct (Figure 1). Circulating B cells then enter distant mucosal tissues, where they are preferentially retained. In these mucosal effector sites, B cells clonally expand and mature into IgA plasma cells^{1-3,6-10}. This cell distribution pathway from IgA inductive tissues (e.g. GALT and BALT) to IgA effector sites (e.g. lamina propria regions of the intestine, the bronchi, the genitourinary tract and secretory glands) has been termed 'the common mucosal immune system'^{1-3,6-10} (Figure 1). In this regard, it was demonstrated that lymphocytes from the PP can repopulate lymphoid tissues of irradiated animals. Particularly important was the finding that the mucosa of the intestinal tract of irradiated rabbits contained IgA-producing cells expressing the L-chain allotypes of the donor rabbit upon transfer of PP lymphocytes⁴. This study was the first to propose that the PP are an enriched source for precursors of IgA-producing cells which possess the capacity to home to mucosal associated tissues. Numerous subsequent studies, performed primarily in mice, extended these observations and the BALT was identified in addition to the GALT as another source of such cells⁵. In addition to the intestinal and respiratory tracts, remote secretory glands such as

mammary, parotid, lacrimal, and cervical glands of the uterus^{7,27,28} can be populated by IgA-precursor cells derived from BALT and GALT.

The importance of PP lymphocytes in dissemination to other exocrine tissues of antigen-sensitized precursors of IgA plasma cells was demonstrated by the use of ligated ileal loops⁶. Two isolated ileal loops, one with and the other without a PP, were constructed without the interruption of the flow of blood and lymph. After the introduction of antigens (dinitrophenyl-coupled keyhole limpet haemocyanin and *Salmonella typhimurium*) into the loop that contained the PP, a S-IgA response was detected in both loops, while these antigens introduced into a loop devoid of PP did not induce an immune response⁶. These observations directly implicated the PP as the source of precursor cells that are capable of undergoing direct antigen-driven stimulation to migrate, divide and differentiate, and repopulate the intestinal lamina propria with cells that secrete specific IgA antibodies. Furthermore, IgA from several secretions of a single animal orally immunized with the dinitrophenyl hapten on a carrier displayed, upon isoelectric focusing, identical spectrotypes, suggesting that IgA cells that populate various exocrine tissues were derived from a common clone⁷.

The existence of the common mucosal immune system (Figure 1), and especially the determination of the origin of mucosal IgA plasma cells in humans is inevitably based on indirect evidence because of the obvious impossibility of performing adoptive transfers of lymphocytes. However, many experiments^{1,10} strongly indicate that an analogous system also operates in humans. Briefly, it was shown that (i) external secretions of glands anatomically remote from the site of antigen stimulation contained S-IgA antibodies that were specific for microbial and food antigens; (ii) ingestion of bacteria and viruses induced the parallel appearance of specific S-IgA in saliva, milk, and tears but not in serum; (iii) specific IgA antibody-forming cells could be transiently detected among peripheral blood mononuclear cells (PBMC) collected 7–12 days after oral immunization and their presence precedes the appearance of specific S-IgA antibodies in saliva and tears; and (iv) analyses of the properties of IgA from stimulated human PBMC revealed that such cells produced predominantly pIgA and display an IgA subclass distribution reminiscent of IgA⁺ cells in mucosal tissues^{1,10}. By these criteria, PBMC differ from IgA-producing cells in the bone marrow, spleen, and lymph nodes; these cells secrete predominantly mIgA of the IgA1 subclass and are J chain-negative^{29,30}.

In summary, one can conclude that in mammals the GALT, especially the PP, are major IgA inductive sites which lead to S-IgA responses in remote external secretions by a cell redistribution pathway termed the common mucosal immune system (Figure 1). It is surprising that despite our current level of understanding of the common mucosal immune system, almost all current vaccines are given to humans by the parenteral route. Systemic immunization is essentially ineffective for induction of mucosal immune responses. Since the majority of infectious microorganisms are encountered through mucosal surface areas, it is logical to consider the induction of protective antibodies and T cell responses in mucosal tissues. The host response via the common mucosal immune system, which occurs to

environmental antigens, should be used to advantage for development of mucosal vaccines.

T cells and cytokines in the mucosal immune system

The induction of effective immunity to pathogenic viruses and bacteria must consider the activation of two well-defined classes of T cells. These include CD3⁺, CD4⁺, CD8⁺ Th cells which are induced to epitopes presented on MHC class II molecules by antigen-presenting cells (APC)^{31,32}. In mucosal inductive sites these APCs include B cells, dendritic cells and macrophages^{1,2}. Activation of antigen-specific CD4⁺ Th cells can lead to secretion of appropriate cytokines (e.g. IL-4, IL-5 and IL-6) for B-cell responses and Ig synthesis. In addition, antigen-activated CD4⁺ Th cells can also up regulate the function of CTLs which are major effectors for elimination of virus-infected cells. CTLs normally express CD8 and respond to virus epitopes presented together with MHC class I molecules^{30,31}. Thus, in order to induce appropriate mucosal immune responses to orally administered vaccines, one must study the induction of antigen-specific CD4⁺ Th and CD8⁺ CTLs in both IgA inductive (e.g. PP) and effector (e.g. intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPL)) sites.

T cell regulation of IgA responses. A direct role for T cells in IgA responses was first shown by experiments with PP T cells activated with concanavalin A (Con A). Addition of activated PP T cells to splenic or PP B cells, triggered with LPS, resulted in four to sixfold increases in IgA synthesis³³; however, IgM and IgG responses were suppressed. On the other hand, Con A activated splenic T cells suppressed IgA synthesis³³. These important findings suggested that a separate T cell subset, enriched in PP but absent in spleen, regulates IgA synthesis. In additional studies, a unique subset of T cells was also discovered in murine PP, which could induce IgA isotype switching^{34,35}. The initial demonstration of switch T cells (Tsw) utilized T-cell clones derived from Con A-stimulated PP cell cultures. Tsw cells from murine PP, in contrast to spleen-derived Tsw cell clones, preferentially induced increased numbers of sIgA⁺ B cells with an accompanying decrease in sIgM⁺ B cells in lipopolysaccharide (LPS)-treated splenic B cell cultures^{34,35}. However, these Tsw cells did not induce terminal differentiation of sIgA⁺ B cells into plasma cells. Other work has shown that a subset of CD4⁺ Th cells in PP could preferentially support antigen-specific IgA responses^{34,35}. In this work, several CD4⁺ Th cell clones were generated from PP of mice orally primed with a classical T cell dependent (TD) antigen, e.g. sheep erythrocytes (SRBC). A subset of PP-derived CD4⁺ Th cells selectively interacted with sIgA⁺ B cells and induced them to become IgA-producing cells³⁶. One of the unique characteristics of these isotype-specific CD4⁺ Th cells was the expression of Fc α receptors (Fc α R), although the exact role of this receptor in isotype-specific immunoregulation is still unknown^{37,38}.

It is now well accepted that cloned murine CD4⁺ Th cells can be separated into two subsets (e.g. Th1 and Th2 cells) according to their profile of cytokine production^{39–41}. In response to activation by foreign antigen, alloantigen or Con A, Th1 cells produce IL-2,

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IFN- γ and lymphotoxin (TNF β) and Th2 cells produce IL-4, IL-5, IL-6 and IL-10³⁹⁻⁴². This subset analysis has been performed at the level of specific mRNA and cytokine synthesis and the bioassay of T cell clones and hybridomas which were kept in long-term culture *in vitro*⁴³. Both classes of antigen-specific Th cell clones express a typical Th cell phenotype, i.e. CD3⁺, CD4⁺, CD5⁺ and CD8⁻. In addition, clones of both Th1 and Th2 cells help B cells respond to antigen although there are important differences in the Ig isotypes produced following cognate- and specific-cytokine interactions. It was recently shown that Th1 and Th2 type CD4⁺ T cells reside in both IgA inductive and effector tissues and the higher expression of Th2 cells in mucosa-associated tissues could be associated with IgA responses that occur in these sites⁴⁴. Using an IFN- γ -(Th1 cells) and IL-5-(Th2 cells) specific ELISPOT assay⁴⁵, the frequencies of Th1 and Th2 type cells were assessed in lymphocyte populations obtained from PP (IgA inductive site), and IEL and LPL (IgA effector site).

Lymphocyte preparations obtained from all mucosa-associated tissues used in this study contained higher numbers of spontaneous IL-5- and IFN- γ -secreting cells when compared with spleen⁴⁴. Among gut-associated tissues, LPL, a major IgA effector site, contained the highest number of IL-5-producing CD4⁺ Th cells with lower numbers of IFN- γ -secreting cells⁴⁴. In contrast, the PP, a major IgA inductive site, possessed fewer IL-5 and IFN- γ -producing CD4⁺ Th cells. In this regard, the frequency of spontaneous IL-5-secreting cells was considerably higher in LPL when compared with PP. Further, freshly isolated CD4⁺ T cells from the LPL subset formed two to three times more IL-5 spot forming cells (SFC) than IFN- γ -producing cells. In contrast, approximately equal numbers of Th1 and Th2 type cells were detected in the PP CD4⁺ T cell fraction, which also had fewer cytokine-producing cells than LPL. These results show that IgA effector sites (LPL) contained higher numbers of IL-5 producing Th2 cells than IFN- γ -secreting Th1 cells, while IgA inductive sites (PP) exhibited an equal distribution of Th1 type and Th2 type cells⁴⁴. Based on these findings, it will be important to determine the frequency of antigen-specific Th1- and Th2-type CD4⁺ Th cell responses in both IgA inductive and effector tissues of mice given vaccines by the oral route.

A significant finding was that IELs also contained both IL-5 and IFN- γ -producing cells⁴⁴ in approximately equal numbers, suggesting that IELs may contain two types of T cells according to their ability to produce either IFN- γ or IL-5. Since the IEL fraction is made up largely of CD8⁺ cells (~80%)⁴⁵ and contains significant numbers of IFN- γ and IL-5 SFC, studies were carried out to determine whether CD8⁺ T lymphocytes in IELs were capable of producing cytokines. IL-5 secreting cells were found in the purified CD8⁺ T-cell fraction of IEL⁴⁴, and similar numbers of CD8⁺ T cells secreting IFN- γ were also seen in the IEL subset. However, very few IFN- γ or IL-5 SFC were seen in CD8⁺ T cells from spleen or PP, showing that CD8⁺ T cells in IEL contain subpopulations which produce both IFN- γ and IL-5. These findings suggested that the IEL population may contain unique subsets of CD8⁺ T cells which could provide Th cell cytokines in addition to a classical function in cytotoxicity. Thus, we can examine the exact role of cytokine-producing CD8⁺ T cells in IELs for both

antigen-specific CTL responses and possible regulatory function for B-cell responses to orally administered antigens.

Finally, it is important to emphasize that enteric immunization with various types of antigens can induce functional effector T cells in PP. Oral priming with SRBC resulted in the induction of Th cells which supported hapten-specific antibody responses⁴⁷. A large number of subsequent studies have shown that Th cells with specificity for soluble⁴⁸ or particulate antigens³⁶⁻³⁸ can be induced in PP by oral immunization. Further, oral administration of BCG induced T cells responsive to purified protein derivative (PPD)⁴⁹. Finally, enteric exposure to reovirus resulted in functional CTLs in the PP¹⁸. A large number of studies have subsequently shown that clonal T cell populations, specific for the enteric antigen, can be derived from the PP (see below). Furthermore, functional Th cells from PP have also been described in detail^{36-38,48}. In conclusion, oral administration of vaccines should be assessed for induction of appropriate antigen-specific CD4⁺ Th cells and CD8⁺ CTLs in both IgA inductive and effector tissues.

Cytokine regulation of IgA responses. Cytokines are of importance in IgA responses, and may be divided into two categories. The first are cytokines which influence the isotype switch to IgA, and the second are those which induce sIgA⁺ B cells to terminally differentiate into IgA plasma cells. Convincing evidence has been presented to show that TGF- β induces sIgM⁺ B cells to switch to sIgA expression^{50,51}. The cytokines IL-5 and IL-6, on the other hand, have been shown to be most effective for induction of IgA synthesis^{2,52,53}.

Several studies have shown that murine IL-5 selectively enhances IgA synthesis^{52,54-58}. It has been shown that an autoreactive T-cell line derived from mouse PP, incubated with splenic LPS-induced B-cell blasts, resulted in enhanced synthesis of both IgG1 and IgA⁵⁴. Furthermore, supernatants from the original cell line, and from one of two daughter cell lines, yielded IL-4 and IL-5 and enhanced IgG1 and IgA synthesis. When added to LPS-driven B-cell cultures, highly purified IL-5 enhanced IgA production and this effect was further increased by IL-4⁵⁴. Other studies have shown that supernatants from Th2 cell clones enhanced IgA synthesis in LPS-triggered splenic B-cell cultures⁵⁵. This IgA-enhancing factor (IgA-EF) was purified from culture supernatants of a Th2 cell clone and a resulting 21-amino acid sequence was found to correspond to that predicted from a cDNA clone of murine IL-5, confirming that the IgA-EF produced by the various T-cell lines was indeed IL-5^{55,56}. The mechanisms of IL-5 regulation of IgA synthesis have been studied in more detail^{52,57,58}, and the results obtained in these studies clearly indicate that IL-5 induces IgA-committed B cells to secrete IgA. Since the PP possess a high frequency of B cells committed to IgA and which express sIgA, PP B-cell subsets have been used to study IL-5-induced effects. In this regard, it was shown that IL-5 induced IgA synthesis in LPS-stimulated sIgA⁺ but not in sIgA⁻ B cells⁵⁷. IL-5 also induced increased numbers of IgA-secreting cells but did not enhance cell division in culture, suggesting that IL-5 induces terminal differentiation of IgA-committed B cells to secrete IgA.

Studies have also been carried out on the target PP B-cell population affected by IL-5 in a system which did

not require LPS stimulation. It is now established that GALT cells are naturally exposed to environmental microbial stimulants and that ≈ 30 – 40% of PP B cells are in cell cycle^{17,52}. It was thus feasible to separate PP B cells into large blasts and small, resting lymphocytes using Percoll gradients. The addition of recombinant IL-5 (rIL-5) to PP B-cell cultures resulted in increased synthesis of IgA, with little or no effect on the production of IgM or IgG isotypes⁵². IL-4 had no effect on IgA synthesis when added either alone or together with IL-5. The IL-5-induced increase in IgA synthesis was restricted to large blast, sIgA⁺ B cells, and the rIL-5-enhanced IgA synthesis occurred in a dose-dependent fashion⁵¹. These studies suggest that IL-5 induces sIgA⁺ B cells to differentiate into cells secreting IgA in a manner completely analogous to the known effects of IL-5 for other isotypes, and that LPS alone may induce B-cell switching to IgA and the increased expression of receptors for IL-5. The presence of IL-5, therefore, would be sufficient to direct the IgA-committed, IL-5R⁺ B cells to become IgA-secreting cells.

It is now well established that IL-6 induces terminal differentiation of B cells which have been activated with mitogen or antigen, and that both rIL-5 and rIL-6, but not rIL-4, induce significant increases in IgA synthesis in PP B-cell cultures⁵³. In these studies, rIL-6 appeared to be two to three times more potent than rIL-5⁵³. Furthermore, both rIL-5 and rIL-6 induced significant increases in IgA levels in the large blast B-cell population; however, the addition of rIL-6 also resulted in IgA synthesis in small, resting B-cell cultures at levels comparable to that induced by rIL-5 in the blast population. When PP B cells were separated into sIgA⁺ and sIgA⁻ B-cell subsets by flow cytometry, removal of sIgA⁺ B cells abolished the effect of both rIL-5 and rIL-6 on IgA synthesis. On the other hand, subsets enriched for sIgA⁺ cells and incubated with rIL-5 or rIL-6 increased IgA synthesis in a dose-dependent manner with rIL-6 appearing to be two to four times more potent than rIL-5. Thus, both IL-5 and IL-6 induce sIgA⁺ blast B-cell subsets to differentiate into IgA-secreting cells. Since IL-6 induces increased numbers of B cells secreting IgA at higher levels of total IgA synthesis, it is more effective for terminal differentiation than IL-5⁵³. Thus, both IL-5 and IL-6 appear to act on B cells already committed to IgA production. In order to evaluate whether orally administered vaccines can induce appropriate IgA responses at mucosal surfaces, one must induce antigen-specific CD4⁺ Th cells which can provide the appropriate cytokines such as IL-5 and IL-6 to antigen-specific sIgA⁺ B cells to induce their differentiation into Ig-producing plasma cells. Further, it is also feasible to consider that the delivery of these cytokines together with specific vaccines to mucosal effector sites could allow the maximum production of antigen-specific IgA responses.

CTLs in mucosal immunity. It is important to consider the induction of effective T-cell immunity at mucosal sites. Generally, this would exist in two forms, namely CTL responses to viral components and induction of CD4⁺ (or possibly CD8⁺) T cells which produce cytokines for activation of macrophages and more effective killing of intracellular bacteria, e.g. *Salmonella*. In this regard, Th1 cells appear to meet this requirement, and are thought to be the principal effector cells for CMI responses. In general, virus-infected cells are lysed by

CD3⁺, CD4⁻, CD8⁺ CTLs which must recognize a complex of virus-derived peptide and major histocompatibility complex (MHC) class I molecules expressed on the surface of infected (target) cells^{31,32}. Since CTLs have been shown to be an effective cellular compartment for the elimination of virus-infected cells, one must evaluate effective CTL responses in mucosa-associated tissues after oral immunization. Studies in both humans and experimental animals have shown that cell-mediated cytotoxicity, antibody-dependent cytotoxicity and natural killer (NK) activity can be associated with mucosal tissues^{46,60–64}. Furthermore, functional CTL activity has also been shown in subsets of IELs and in LPLs^{46,64–66}. In addition to antigen-specific CTLs, IELs contain a subset with NK function and this also exhibits spontaneous cytotoxic activity^{46,67}. It is important to indicate that antigen-specific CTLs can be generated in IgA inductive sites such as the PP by oral administration. Thus, CTL function directed to mismatched MHC was induced in PP of mice given allogeneic tumour cells via the oral route^{68,69}. More importantly, it was shown that alloantigen-specific CTLs were induced in IELs isolated from mice orally immunized with allogeneic tumour cells^{70,71}. When CTL clones were established from IELs of alloantigen orally immunized mice, these clones could be separated into two subsets⁷¹. One population of cloned CTLs exhibited classical characteristics of CD8⁺ cytotoxic T cells with respect to proliferation and cytolytic activity. On the other hand, the second subset of T cells possessed unique properties in which high concentrations of IL-2 could activate these cells to exhibit non-antigen-specific lytic potential including NK activity⁷¹. These findings clearly suggest that mucosa-associated tissues are also protected by cytolytic mechanisms including CTLs, antibody-dependent cell cytotoxicity (ADCC) and NK cells in addition to S-IgA antibodies.

Several recent studies have indicated that IELs could play important roles in the induction and regulation of mucosal immune responses. IELs possess a number of unique features which are distinct from lymphocytes residing in other lymphoid tissues. For example, IELs consist of four distinct subsets of T cells including CD4⁻, CD8⁺, ($\approx 75\%$), CD4⁺, CD8⁻ ($\approx 7.5\%$), CD4⁻, CD8⁻ (double negative, DN $\approx 7.5\%$) and CD4⁺, CD8⁺ (double positive, DP $\approx 10\%$)⁷¹. In mice, ≈ 50 – 60% of CD3⁺ T cells in IELs express the T cell receptor (TCR) composed of a γ -chain disulphide linked to the δ chain^{72–74}. Thus, IELs are enriched for γ/δ TCR⁺ T cells which are not seen in other organized lymphoid tissues. Further, the uniqueness of CD3⁺, γ/δ TCR⁺ T cells in IELs is derived from the finding that the antigen receptor of these γ/δ T cells is predominantly encoded by the distinct V $_{\gamma 7}$ gene⁷⁴. It is essential to examine the exact functions and characteristics of CD3⁺ T cells in IELs of mice which have been orally immunized with various vaccines.

Finally, it is important to note that oral administration of antigens, e.g. virus, can induce virus-specific CTLs in GALT as well as in other mucosa-associated tissues. Oral immunization of rats with vaccinia resulted in the generation of virus-specific CTLs in PP and mesenteric lymph nodes (MLN)⁷⁵. Vaccinia-specific CD4⁻, CD8⁺ CTLs were found in the MLN within 1 week of oral immunization. This would suggest that after enteric immunization, antigen-stimulated CTLs were dissem-

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inated from PP into MLN via the lymphatic drainage⁷⁴. Further, virus-specific CTLs were also induced in mucosa-associated tissues by oral administration of reovirus and with rotavirus^{18,76}. In these studies, a high frequency of virus-specific CTLs was present in the IgA inductive tissues as early as 6 days after oral immunization. It should also be noted that oral immunization induced antigen-specific CTLs in systemic tissues (e.g. spleen) in addition to mucosa-associated tissues⁷⁶. Based on these findings, it is likely that oral immunization with viral antigens induces antigen-specific CTLs in both IgA inductive and effector sites for mucosal responses and possibly in systemic lymphoid tissues as well. Induction of effective mucosal CTL responses to virus would be advantageous to the host, since mucosal elimination of virus invasion should be considered as a first line of immune defence to prevent subsequent systemic infection.

FUNCTIONS OF S-IgA ANTIBODIES

IgA in external secretions occurs predominantly in dimeric and tetrameric forms (with four or eight antigen-binding sites, respectively) and thus displays greater avidity than monomeric Ig. Polymeric IgA has been shown to neutralize viruses more effectively than monomeric IgA^{77,78}, and its multivalence also enables Ig polymers to agglutinate bacteria better than corresponding monomers. In addition to viruses, S-IgA antibodies also neutralize other biologically active antigens such as bacterial toxins and enzymes⁷⁹. IgA functions in an environment rich in proteolytic enzymes⁸⁰. Its intrinsic resistance to proteolysis, further reinforced by association with secretory component (SC), preserves many biological functions of intact Ig molecules, and provides functional advantages to IgA when compared with antibodies of other isotypes that may also be found on mucosal surfaces^{1,79}.

Virus neutralization by IgA

Virus-neutralizing antibodies at mucosal surfaces are important in preventing local infection and disease⁸¹. Although neutralizing antibodies of IgA, IgG, and IgM isotypes to several pathogenic viruses have been described, little is actually known regarding the precise mechanism(s) involved in viral inactivation, which has come to be known as neutralization⁷⁷. The binding of a single antibody molecule to a viral particle ('single-hit') may result in inactivation⁷⁷. S-IgA antibodies are particularly effective in virus neutralization, perhaps due in part to the presence of four (or eight) antigen binding sites. Furthermore, S-IgA antibodies play an important role in antiviral immunity, because they are present at the sites of initial contact of virions with host cells. The view that has emerged is that S-IgA in the mucus layer can prevent virus attachment to epithelial cells and thus block their subsequent penetration; however, this view may be too simplistic⁸². Viruses possess specific structural protein subunits of importance in attachment and infection. For example, the influenza virus surface contains subunits of two different proteins, haemagglutinin (HA) and neuraminidase (NA), but only HA is involved in cell attachment⁸³ and corresponding antibodies effectively prevent virus infection.

There are some 700–1200 HA spikes on the influenza virion and approximately 50 molecules of monoclonal IgG antibody to the virus can neutralize 50% of the

infectious dose⁸⁴. Under these conditions, a high number of HA spikes remain unbound, and the type A influenza virus has been shown to attach and to enter various host cells even after neutralization^{77,85}. Both neutralized and untreated influenza virions enter the cell through the plasma membrane and reach the nucleus⁷⁷, but virus-specific mRNA is not transcribed from neutralized virions⁸⁵. The mechanism for this may involve a conformational change in HA following antibody binding, which results in transmission of a signal to the transcriptase complex inside the virus envelope membrane⁷⁷. The epithelial cells infected by virus in the upper respiratory tract are involved with IgA transport, and are therefore prone to antigen-antibody interaction that prevents viral nucleotide transcription.

It is often said that the unique characteristics of S-IgA, including its polymeric nature, avidity for mucus, and resistance to proteolytic enzymes, all contribute to the apparent efficiency of this antibody isotype in virus neutralization. The importance of the molecular form of IgA in virus neutralization has been emphasized in studies with influenza A⁸⁴. Rat S-IgA (collected from bile) was dissociated into IgA monomers, without the loss of specificity or titre, for direct comparison with intact S-IgA (and serum IgG) for neutralization efficiency. S-IgA anti-HA antibodies prevented virus attachment, while monomeric IgA and IgG allowed attachment, penetration and viral genome accumulation in the cell nucleus⁸⁴. These results suggest that S-IgA may sterically block HA on influenza A virus and prevent its entry into the cell.

Antibacterial functions

Mucosal antibodies have been shown to inhibit microbial adherence and prevent absorption of antigens from mucosal surfaces. The direct inhibitory effect of S-IgA on the adherence of micro-organisms to host mucosal epithelial cells has been documented in many experimental systems^{86,87}. Both non-specific hydrophobic interactions as well as specific inhibition of binding between bacterial surface adhesins and complementary surface receptors on host epithelial cells are involved in this process. In this respect, IgA and especially S-IgA are more effective than IgG antibodies of the same specificity owing to their unique charge, extensive glycosylation, and their resistance to proteolysis⁸⁰. IgA can inhibit adherence of members of the *Enterobacteriaceae* family to epithelial cells by mechanisms that are independent of specific antibody activity. Terminal mannose-containing oligosaccharide side chains on the heavy chain, present especially on the IgA₂ molecules, are recognized by mannose-specific lectins present on type I fimbriae. Thus, these carbohydrate-specific interactions represent an important protective anti-adherence function of S-IgA against a broad spectrum of bacteria, regardless of the specificity of the IgA molecule. The effectiveness of S-IgA *in vivo* is further amplified by a number of other factors such as the interactions with innate immune components, glycoproteins, the continuous desquamation of epithelial cells with attached bacteria, and the intense competition with other colonizing bacteria, as demonstrated by immunization with *Vibrio cholerae* of mono-associated or conventional animals^{79,88,89}. Apparently, a bacterium selectively disadvantaged by the specific S-IgA is more effectively displaced by other bacteria in this competitive environment.

Several studies have shown that IgA of serum as well

as secretory origin is able to potentiate the function of some of the innate anti-bacterial factors present in external secretions^{79,88}. Thus, the antimicrobial effects of lactoferrin and the lactoperoxidase system against several mucosal pathogens can be further enhanced by secretory or serum IgA^{79,88}.

IgA of various molecular properties can interact with a broad spectrum of morphologically and functionally diverse cells that display receptors for the various structural components in the Fc region of the molecule¹. Although these interactions have important biological consequences (including selective transport into external secretions, IgA catabolism, and enhancement or suppression of the immune response), interactions with mucosal phagocytic cells and lymphocytes involved in the ADCC may be of special relevance. The antibacterial activity of monocytes and lymphocytes from mucosal as well as peripheral lymphoid organs can be enhanced by S-IgA in the ADCC assays⁹⁰. The latter population of cells, subsequently characterized as CD3⁺, CD4⁺, and Leu 8⁺ subset of T-lymphocytes, can bind IgA through the Fc region, and such 'armed' cells can effectively kill bacteria of *Salmonella* and *Shigella* species⁹⁰. The increased numbers of T cells that participated in IgA-ADCC were seen in individuals orally immunized with *Salmonella* Ty21a vaccine⁹⁰.

Immune exclusion

Extensive studies have demonstrated that the previous enteric exposure to foreign soluble antigens diminishes the absorption of the same but not of unrelated antigens owing to the presence of specific S-IgA antibodies^{79,91,92}. Through this immune exclusion mechanism, IgA limits further absorption of undigested antigenic material and the formation of potentially harmful circulating immune complexes that contain predominantly IgG antibodies as seen in the IgA-deficient individuals⁹³. The inability of human secretory or serum IgA complexed to antigens to activate complement by either classical or alternative pathways may be of paramount importance in the maintenance of integrity of mucosal surfaces. Although an encounter of IgA antibody with the corresponding antigen in the mucosal surfaces blocks the absorption of such antigen, it does not result in complement activation followed by the generation of cleavage products of the C3 and C5 components. These fragments would induce local inflammatory reactions, including the influx of polymorphonuclear leucocytes and release of substances that enhance the permeability of mucosal membranes due to tissue damage.

The unfavourable biological consequences of antigen encounter with a corresponding antibody of inflammatory isotype have been well documented: although the absorption, from mucosal surfaces, of an antigen may also be inhibited by corresponding IgG antibody, such immune complexes formed in the mucosal tissues activate complement, and the resultant local damage leads to increased absorption of bystander antigens^{94,95}. Moreover, IgA antibodies may also abrogate anaphylactic and Arthus type reactions mediated by inflammatory antibodies of IgE and IgG isotypes^{96,97}. Thus, it seems that the major biological role of IgA antibody at mucosal surfaces is the mitigation of the inflammatory side effects brought about by other immune effector mechanisms.

MUCOSAL ANTIGEN DELIVERY SYSTEMS

Both soluble and particulate antigens have been used extensively for the induction of secretory immune responses in several animal models and in humans¹. Although some protein or glycoprotein antigens such as cholera toxin⁹⁸⁻¹⁰⁰, ricin, HA of influenza virus¹⁰¹⁻¹⁰³ and others¹⁰⁴ can easily induce measurable antibodies in secretions and in serum of orally immunized subjects, it is generally accepted that much higher and more frequently administered oral doses of antigens are necessary than with systemic immunization. The response to orally administered antigen is low because few undigested and fully immunogenic antigens reach the immune system of the intestine, most having been either degraded by enzymes or only partially absorbed because of their high molecular weight¹⁰⁵. Particulate antigens, most notably micro-organisms such as viruses and bacteria that multiply within the lumen of the intestine or multiply even better in the GALT, provide a more effective stimulus for the induction of a local as well as a generalized secretory and systemic immune response^{10,104}.

To preserve the immunogenic material from acid pH and proteolysis, orally administered antigens have been given with large amounts of sodium bicarbonate^{10,106}. Another more effective approach involved packaging of bacterial or viral antigens in gelatin capsules that were then coated with substances soluble only at the alkaline pH of the small intestine^{101,102}. Other studies, limited in number and briefly summarized here, have considered the use of substances that may function as oral adjuvants and thus enhance, by various mechanisms, both secretory and systemic immunity.

Cholera toxin and CT-B subunit conjugates

Cholera toxin (CT) produced by *Vibrio cholerae* binds to various nucleated cells, including intestinal epithelium, through a specific receptor GM1 ganglioside^{98,99,107,108}. Because of the high affinity of CT for GM1-expressing cells, oral immunization induces a highly specific and vigorous immune response manifested by S-IgA and serum IgG antibodies with extended immunological memory¹⁰⁷ (Figure 1).

Structure. Cholera toxin has been extensively characterized as a result of its importance in the virulence of *Vibrio cholerae*¹⁰⁹. It is composed of an A and B subunit. The toxinogenic A subunit (28 kDa) is involved in the ADP-ribosylation of the adenylate cyclase regulatory protein Gs. This modification increases cAMP levels, and causes intestinal secretion, resulting in the diarrhoea and fluid loss observed in patients with cholera. The A subunit (CT-A) is post-translationally cleaved into the A1 and A2 peptides, and it is the A1 peptide that is toxinogenic. It is hypothesized that the A2 peptide mediates the association of the A1 peptide with the binding subunits. The B subunit of cholera toxin (CT-B) is composed of five identical non-covalently associated subunits (11.6 kDa) which serve as the carrier for the A subunit. The CT-B pentamer binds to the monosialoganglioside GM1¹¹⁰, which is present on all nucleated cells and is found in abundance on the surface of intestinal epithelial cells. Such membrane binding induces a conformational change that allows the A subunit to penetrate into the cell¹⁰⁹.

Cholera toxin as an immunogen. The immune response to CT has been exhaustively studied, due to the ability of neutralizing antibodies to reduce the severity of the disease. During these studies, it became clear that CT was one of the most potent oral immunogens ever identified¹¹¹, inducing strong antibody responses when administered into the GI tract in microgram amounts. The antibody response consists not only of a specific S-IgA response but also a plasma IgG response, both of which appear to originate in the GALT¹⁷. Thus the immunity extends not only to the mucosal surface but also to the systemic compartment. The feeding of most other protein antigens, even in milligram doses, not only fails to induce mucosal responses but also induces a state of unresponsiveness which is evident when one subsequently immunizes parenterally (oral tolerance). This is the reverse of what is desired for oral vaccines and is a potential pitfall for their development. CT and CT-B are among the few proteins that do not induce oral tolerance¹¹², again a very desirable property. The genetic background of the host is important, in that both the secretory IgA and plasma IgG responses to CT are restricted by the I-A subregion of the H-2 major histocompatibility locus^{113,114}. The antibody response appears to regionalize, i.e. the plasma cell response is most abundant at mucosal sites that were directly exposed to CT¹¹⁵. Exploitation of this feature by combined local mucosal-oral immunizations may allow the major IgA response to occur in selected non-intestinal sites. Lastly, oral immunization with CT results in prolonged memory responses in the intestinal lamina propria, and presumably at other mucosal sites¹¹⁶. These properties have made CT a model antigen for probing the mucosal immune system, but they also represent the properties one would desire in an ideal oral vaccine. Because some of the strategies of using CT as an oral adjuvant involve its physical coupling to another antigen, the resulting neoantigen should retain some of the desirable properties of CT itself.

Cholera toxin as an adjuvant. To determine whether CT feeding resulted in oral tolerance, Elson and Ealding¹¹² demonstrated the ability of CT to abrogate oral tolerance to keyhole limpet haemocyanin (KLH) when mice were fed both proteins together. Such feeding of CT with KLH not only abrogated tolerance, but also induced an intestinal S-IgA response to KLH which did not occur when KLH was fed alone. This suggested that feeding CT together with antigens that are normally poor oral immunogens may elicit mucosal immune responses to such antigens. The ability of CT to act as an oral adjuvant has since been confirmed by a number of other investigators with a variety of antigens^{112,117-125}.

CT does not fit the classical definition of an adjuvant, because it stimulates an immune response to itself and because the adjuvanticity of CT may be related to and dependent upon its immunogenicity: the response of mice to KLH given together with CT orally was significantly higher in mouse strains that are high responders to CT than in H-2 congenic strains that were low responders to CT¹²⁶. However, because CT can enhance the immune response to other antigens, it shares one of the most important properties of an adjuvant. CT possesses the necessary B- and T-cell epitopes required to elicit systemic immunity and, as such, it has been used

effectively as a carrier for several parenterally administered experimental vaccines¹²⁷⁻¹²⁹. It may be that, given orally, CT provides the necessary signals that alter the regulatory environment of the GALT from one of suppression to responsiveness. The precise mechanism of action is not known.

The ability of CT to stimulate immunity to unrelated antigens depends upon several factors. In order to induce immunity to the target antigen, CT has to be administered simultaneously with the antigen¹³⁰. In addition, both the antigen and CT must be administered by the same route, i.e. orally. Giving each protein by a different route is not effective. This suggests that CT alters the mucosal lymphoid tissue in a manner that favours responsiveness to the antigens presented to it. The genetic responsiveness of the host for the antigen and for CT is important in that the adjuvant effect of CT appears to be genetically restricted¹²⁶, as mentioned above. The dose of CT required for the adjuvant effect is not clearly defined, and may vary depending on the antigen involved. Although very small amounts of CT sufficed to potentiate the immune response to CT-B, much larger amounts have been used for the adjuvant effect for viruses. The length of the memory response to the antigen induced by CT is not known. Currently, no one has studied the long-term immunological responses of any CT-enhanced antigens, although CT does induce long-term immunological memory to itself¹¹⁶. The adjuvant effect observed for CT will also probably depend on the type of antigen administered. CT has been used with success orally with proteins and viruses, and parenterally as a vaccine carrier for polysaccharides¹³¹. Despite these successes, we have been unable to stimulate S-IgA responses to ovalbumin when it is given orally with CT (Elson, unpublished observation). Therefore the adjuvanticity of CT may not apply to all antigens. The suitability of a given antigen for this vaccination method will probably have to be empirically determined.

Although CT enhances the secretory and systemic antibody responses elicited to antigens given orally (Figure 1), it is not known whether similar effects would occur in humans. However, from a practical standpoint, the use of holotoxin is not feasible due to its toxicity. One approach being explored to resolve this problem is to use the non-toxic B subunit instead. Apart from being non-toxic, CT-B stimulates good immunity to itself when given orally, which has raised hopes for its use as a vaccine adjuvant instead of the holotoxin. McKenzie and Halsey¹¹⁷ were the first to report the use of CT-B in such a role. In their experiment, CT-B was chemically coupled to horseradish peroxidase (HRP). The results showed that HRP conjugated to CT-B elicited higher antibody levels in the gut and serum than those obtained by feeding HRP alone or as an unconjugated mixture of HRP and CT-B. Since then, the use of CT-B as a vaccine adjuvant has been examined by others with variable success. Lycke and Holmgren¹³⁰ were unable to stimulate immunity to KLH when mixed with CT-B unless they added very small doses (< 50 ng) of holotoxin. However, they did not use a conjugated form of KLH and CT-B. The use of CT-B conjugates has been reported to be effective in some cases^{118,119} but not in others^{120,121}. The poor responses that have been observed with CT-B conjugates in some cases may be due to the coupling procedure. The degree of cross linking and coupling procedure used can significantly affect the immunogenicity of protein con-

jugates¹³². Further experimentation is necessary in order to determine whether CT-B can replace the holotoxin as an adjuvant.

The use of CT as a mucosal adjuvant is a promising new approach to oral vaccination. However, much remains to be learned about the mechanism of both the immunogenicity and adjuvanticity of CT, including the relative contributions of the two subunits. The dose, timing and genetic background of the host are all important variables. There are indications that the mechanism of the adjuvanticity of CT involves multiple aspects of immune induction in the mucosa, including increased uptake of antigen into mucosal follicles; enhancement of IL-1 production by APCs; altered regulation by T cells, especially inhibition of CD8⁺ suppressor cells; stimulation of B-cell switching to IgA and IgG; and possibly enhancement of B-cell clonal expansion. Different components of these multiple effects may be of more importance for some antigens than others.

Biodegradable microspheres

Recently, various antigens have been incorporated without chemical modification into biodegradable microspheres^{133,134} to be used in oral and systemic immunization. Microencapsulation involves the coating of a bioactive agent, such as a vaccine, in a protective wall material which is polymeric in nature. The microsphere product is a free-flowing powder of spherical particles which can be produced in a size range from $\leq 1 \mu\text{m}$ to as large as 3 mm in diameter. The particular system of interest for studies with vaccines involves the use of poly(DL-lactide-co-glycolide) (DL-PLG) copolymers¹³⁵⁻¹³⁷. These biocompatible polyesters are currently used in resorbable sutures and biodegrade *in vivo* into lactic and glycolic acids through the hydrolysis of ester linkages. The rate of degradation, and thus antigen release, is determined by the ratio of lactide to glycolide present in the copolymer and may be from a few days to 2 years¹³⁸.

Vaccine antigens (e.g. staphylococcal enterotoxin B) injected in the form of 1–10 μm microspheres composed of equal mole parts of polymerized lactide and glycolide (50:50 DL-PLG) stimulate up to 500-fold higher plasma antibody responses than that induced by the free antigen^{139,140}. When administered orally, the microspheres protect the encapsulated antigen from acid and proteolytic digestion in the gastrointestinal tract, and those of $\leq 10 \mu\text{m}$ are readily taken up by the M cells of the PP and transported into the T- and B-cell zones^{133,139,141,142}. Those microspheres $< 5 \mu\text{m}$ are ingested by MAC-1 positive cells and transported to systemic lymphoid tissues such as the spleen where the released antigen stimulates a serum antibody response. In contrast, the microspheres $> 5 \mu\text{m}$ remain in the PP, and provide a sustained release of antigen in this IgA inductive tissue. Our studies have shown that microspheres provide several advantages which make them an attractive vaccine delivery system^{133,139-142} (Figure 1). This immunization method concurrently induces systemic and secretory antibody production and thus provides an adjuvant system composed of pharmaceutically approved components with which to determine what role both circulating and mucosal antibodies play in protection against a variety of infectious agents. In addition, it is important to investigate the efficacy with which this

immunization method induces CMI responses by splenic and intraepithelial CD8⁺ lymphocytes against syngeneic target cells expressing vaccine antigen.

In addition to the increased immunogenicity due to the adjuvant effect of antigen delivery in microspheres, plus slow or fast antigen release, there are many other potential advantages to this method of immunization. Because the microspheres do not dissolve immediately upon ingestion, incorporated antigens remain protected from digestion by proteolytic enzymes. In contrast with the killed or live bacteria, whose adherence or uptake by PP can be inhibited by pre-existing antibodies, the absence of antigens on the surface of microspheres may confer an important advantage in this respect. Appropriate mixtures of fast- and slow-releasing microspheres may also induce the primary and multiple booster immune responses with only a single immunization. In the absence of potent adjuvants suitable for use in human medicine, antigens incorporated in biodegradable, non-inflammatory microspheres can significantly improve the immunogenicity of a number of currently available, injectable vaccines. This new technology is likely to substantially influence the design of vaccines for both oral and systemic immunizations¹⁴³.

Selective delivery of antigens by recombinant bacteria

The ability of *Salmonella typhi* or *S. typhimurium* to invade, persist and proliferate in the human or murine GALT, liver and spleen has been known for many years¹⁴⁴. These findings prompted several investigators to use attenuated, genetically modified strains of *Salmonella* as vectors for the selective delivery to the GALT of other antigens produced by such strains as a result of the introduction of coding genes (Figure 1). For example, recombinant avirulent *S. typhimurium* that expressed surface protein A or glucosyltransferase of *S. mutans* was used to induce both secretory and systemic immune responses as well as protective immunity to dental caries in rats^{144,145}. Other mutants of *Salmonella* have been used for the expression of foreign antigens such as *Shigella sonnei* O-antigen^{1,103}, fimbriae, β -galactosidase or heat-labile enterotoxin B subunit of *Escherichia coli*, M protein of *Streptococcus equi*¹⁴⁴ or M protein of *Streptococcus pyogenes*¹⁴⁶ or membrane proteins of *Francisella tularensis*¹⁴⁷. Concomitant appearance of secretory and serum IgA, IgM and IgG antibodies ensued as a consequence of colonization of the gastrointestinal tract with orally administered recombinant *Salmonella*.

Much effort has been devoted to rendering *Salmonella* strains avirulent while preserving their colonizing ability, so that they can induce a protective immune response when later challenged with fully virulent strains. Although mutants devoid of virulence-coding plasmids have fulfilled some of these criteria when given by the oral route, high doses of bacteria given orally or injected intraperitoneally may lead to the death of infected animals. Thus, the elimination of a virulence plasmid would be insufficient for the development of a safe vaccine.

In an alternative approach, several investigators generated auxotrophic mutants, which rendered *Salmonella* avirulent without preventing immunogenicity¹⁴³. Such mutants were unable to synthesize p-aminobenzoic acid, purines, aromatic amino acids, threonine, methionine, diaminopimelic and dihydroxybenzoic acids, or any combination thereof, and so became totally or

partially avirulent, and could not survive outside the host, but retained their immunogenicity. Other mutants were altered in their ability to utilize and synthesize carbohydrates and certain amino acids, or proliferate at temperatures above 37°C. Particularly promising as suitable vectors are novel *Salmonella* mutants that are genotypically stable, completely avirulent, highly immunogenic and are capable of expressing virulence antigens from other pathogens and of displaying gene defects that are not remediable by diet or by the host (Δ cya Δ crp Δ and *Salmonella* strains)^{144,145}. Thus, new vaccines will soon be designed and constructed that will give a long-lasting immunity to the pathogen supplying the genetic information for virulence antigens and to *Salmonella*. However, induction of the mucosal immune response to *Salmonella* may restrict its repeated use for boosting or as a common vector for multiple oral immunizations. Several constructs of salmonella that express virus antigens have been produced and tested in experimental models. For example salmonella expressing antigens of hepatitis B, dengue, herpes simplex, or rotaviruses have been developed¹⁴⁸⁻¹⁵¹. Other bacteria and viruses also have been used or are being considered as suitable live vectors for delivery of antigens to stimulate the mucosal and systemic immune response. For example, oral vaccines based on recombinant strains of *Escherichia coli*, *Yersinia enterocolitica*, lactobacilli or bacterium Calmette-Guerin (BCG) have been tested in animals¹⁵²⁻¹⁵⁶. Viruses that infect the gastrointestinal tract, such as polio or adenovirus, are also considered as potential vectors that may express antigens of other viruses.

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REFERENCES

- Mestecky, J. and McGhee, J.R. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv. Immunol.* 1987, **40**, 153
- McGhee, J.R., Mestecky, J., Elson, C.O. and Kiyono, H. Regulation of IgA synthesis and immune response by T cells and interleukins. *J. Clin. Immunol.* 1989, **9**, 175
- Hanson, L.Å. and Brandtzaeg, P. The mucosal defence system. In: *Immunological Disorders in Infants and Children* (Ed. Stiehm, E.R.) W.B. Saunders Co., Philadelphia, 1989, p. 116
- Craig, S.W. and Cebra, J.J. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. *J. Exp. Med.* 1971, **134**, 188
- Rudzick, R., Clancy, R.L., Perey, D.E., Day, R.P. and Bienenstock, J. Repopulation with IgA-containing cells of bronchial and intestinal lamina propria after transfer of homologous Peyer's patches and bronchial lymphocytes. *J. Immunol.* 1975, **114**, 1599
- Robertson, S.M. and Cebra, J.J. A model for local immunity. *Ric. Clin. Lab.* 1976, **6** (Suppl 3), 105
- Montgomery, P.A., Ayyildiz, A., Lemaitre-Coelho, I.M., Vaerman, J.-P. and Rockey, J.H. Induction and expression of antibodies in secretions: the ocular immune system. *Ann. NY Acad. Sci.* 1983, **409**, 428
- Scicchitano, R., Stanisz, A., Ernst, P.E. and Bienenstock, J. A common mucosal immune system revisited. In: *Migration and Homing of Lymphoid Cells*, Volume II (Ed. Husband, A.J.) CRC Press, Boca Raton, FL, 1988, p. 1
- Phillips-Quagliata, J.M. and Lamm, M.E. Migration of lymphocytes in the mucosal immune system. In: *Migration and Homing of Lymphoid Cells*, Volume II (Ed. Husband, A.J.) CRC Press, Boca Raton, FL, 1988, p. 53
- Mestecky, J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.* 1987, **7**, 265
- Tomasi, T.B. Jr. Oral tolerance—A review. *Transplantation* 1980, **29**, 353
- Bienenstock, J. Bronchus-associated lymphoid tissue. In: *Immunology of the Lung and Upper Respiratory Tract* (Ed. Bienenstock, J.) McGraw-Hill, New York, NY, 1980, p. 96
- Bockman, D.E. and Cooper, M.D. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix and Peyer's patches. *Am. J. Anat.* 1973, **136**, 455
- Owens, R.L. and Jones, A.L. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* 1974, **66**, 189
- Wolf, J.L. and Bye, W.A. The membranous epithelial (M) cell and the mucosal immune system. *Annu. Rev. Med.* 1984, **35**, 95
- Butcher, E.C., Rouse, R.V., Coffman, R.L., Nottenburg, C.N., Hardy, R.R. and Weissman, I.L. Surface phenotype of Peyer's patches germinal center cells: implications for the role of germinal centers in B-cell differentiation. *J. Immunol.* 1982, **129**, 2698
- Lebman, D.A., Griffin, P.M. and Cebra, J.J. Relationship between expression of IgA by Peyer's patch cells and functional IgA memory cells. *J. Exp. Med.* 1977, **166**, 1405
- London, S.D., Rubin, D.H. and Cebra, J.J. Gut mucosal immunization with reovirus serotype 1/L stimulates virus-specific cytotoxic T cell precursors as well as IgA memory cells in Peyer's patches. *J. Exp. Med.* 1987, **165**, 830
- Brandtzaeg, P. Research in gastrointestinal immunology. State of the art. *Scand. J. Gastroenterol.* 1985, **14** (Suppl.), 137
- Kanof, M.E., Strober, W. and Fiocchi, C. CD4-positive, Leu 8-negative helper-inducer T cells predominate in the human intestinal lamina propria. *J. Immunol.* 1988, **141**, 3029
- Bull, D.M. and Bookman, M.A. Isolation and functional characterization of human mucosal lymphoid cells. *J. Clin. Invest.* 1977, **59**, 966
- Solvason, N., Lehuen, A. and Kearney, J.F. An embryonic source of Ly1 but not conventional B cells. *Int. Immunol.* 1991, **3**, 543
- Kroese, G.F.M., Butcher, E.C., Stall, A.M., Lalor, P.A., Adams, S. and Herzenberg, L.A. Many of the IgA producing plasma cells in the murine gut are derived from self-replenishing precursors in the peritoneal cavity. *Intern. Immunol.* 1988, **1**, 75
- Herzenberg, L.A., Stall, A.M., Lalor, P.A., Sidman, C., Moore, W.A., Parks, D.R. and Herzenberg, L.A. The Ly-1 B cell lineage. *Immunol. Rev.* 1986, **93**, 81
- Hayakawa, K., Hardy, R.R., Honda, M., Herzenberg, L.A., Steinberg, A.D. and Herzenberg, L.A. Ly-1 B cells: Functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc. Natl. Acad. Sci. USA* 1984, **81**, 2494
- Hayakawa, K., Hardy, R.R. and Herzenberg, L.A. Peritoneal Ly-1 B cells: genetic control, autoantibody production, increased lambda light chain expression. *Eur. J. Immunol.* 1986, **16**, 450
- Roux, M.E., McWilliams, M., Phillips-Quagliata, J., Weisz-Carrington, P. and Lamm, M.E. Origin of IgA-secreting plasma cells in the mammary gland. *J. Exp. Med.* 1977, **146**, 1311
- McDermott, M.R. and Bienenstock, J. Evidence for a common mucosal system. I. Migration of B immunoblasts into intestinal, respiratory and genital tissues. *J. Immunol.* 1979, **122**, 1892
- Brandtzaeg, P. Immunohistochemical characterization of intracellular J-chain and binding site for secretory component (SC) in human immunoglobulin (Ig)-producing cells. *Mol. Immunol.* 1983, **20**, 941
- Brandtzaeg, P. The role of J chain and secretory component in receptor-mediated glandular and hepatic transport of immunoglobulins in man. *Scand. J. Immunol.* 1985, **22**, 111
- Marrack, P. and Kappler, J. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. *Adv. Immunol.* 1986, **38**, 1
- Bierer, B.E., Sleckman, B.P., Ratnoffsky, S.E. and Burakoff, S.J. The biologic roles of CD2, CD4 and CD8 in T-cell activation. *Annu. Rev. Immunol.* 1989, **7**, 579
- Elson, C.O., Heck, J.A. and Strober, W. T-cell regulation of murine IgA synthesis. *J. Exp. Med.* 1979, **149**, 632
- Kawanishi, H., Saltzman, L.E. and Strober, W. Characteristics and

- regulatory function of murine Con A-induced, cloned T cells obtained from Peyer's patches and spleen: Mechanisms regulating isotype-specific immunoglobulin production by Peyer's patch B cells. *J. Immunol.* 1982, **129**, 475
- 35 Kawanishi, H., Saltzman, L. and Strober, W. Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. I. T cells derived from Peyer's patches that switch sIgM B cells to sIgA B cells *in vitro*. *J. Exp. Med.* 1983, **157**, 433
- 36 Kiyono, H., Mosteller, L.M., Eldridge, J.H., Michalek, S.M. and McGhee, J.R. IgA responses in *xid* mice: Oral antigen primes Peyer's patch cells for *in vitro* immune-responses and secretory antibody production. *J. Immunol.* 1983, **131**, 2616
- 37 Kiyono, H., McGhee, J.R., Mosteller, L.M., Eldridge, J.H., Koopman, W.J., Kearney, J.F. and Michalek, S.M. Murine Peyer's patch T-cell clones: Characterization of antigen-specific helper T cells for immunoglobulin A responses. *J. Exp. Med.* 1982, **156**, 1115
- 38 Kiyono, H., Cooper, M.D., Kearney, J.F., Mosteller, L.M., Michalek, S.M., Koopman, W.J. and McGhee, J.R. Isotype-specificity of helper T-cell clones. Peyer's patch Th cells preferentially collaborate with mature IgA B cells for IgA responses. *J. Exp. Med.* 1984, **159**, 798
- 39 Mosmann, T.R. and Coffman, R.L. Two types of mouse helper T-cell clone: Implication for immune regulation. *Immunol. Today* 1987, **8**, 223
- 40 Mosmann, T.R. and Coffman, R.L. Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 1989, **7**, 145
- 41 Coffman, R.L., Seymour, B.W.P., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Shrader, B. *et al.* The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* 1988, **102**, 5
- 42 Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L. Two types of murine helper T-cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 1986, **136**, 2348
- 43 Cherwinski, H.M., Schumacher, J.H., Brown, K.D. and Mosmann, T.R. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays and monoclonal antibodies. *J. Exp. Med.* 1987, **166**, 1229
- 44 Taguchi, T., McGhee, J.R., Coffman, R.L., Beagley, K.W., Eldridge, J.H., Takatsu, K. and Kiyono, H. Analysis of Th1 and Th2 cells in murine gut-associated tissues. Frequencies of CD4⁺ and CD8⁺ T cells that secrete IFN- γ and IL-5. *J. Immunol.* 1990, **145**, 68
- 45 Taguchi, T., McGhee, J.R., Coffman, R.L., Beagley, K.W., Eldridge, J.H., Takatsu, K. and Kiyono, H. Detection of individual mouse splenic T cells producing IFN- γ and IL-5 using the enzyme-linked immunospot (ELISPOT) assay. *J. Immunol. Methods* 1990, **128**, 65
- 46 Ernst, P.B., Befus, A.D. and Bienenstock, J. Leukocytes in the intestinal epithelium: an unusual immunological compartment. *Immunol. Today* 1985, **6**, 50
- 47 Kagnoff, M.F. Functional characteristics of Peyer's patch cells III. Carrier priming of T cells by antigen feeding. *J. Exp. Med.* 1975, **142**, 1425
- 48 Richman, I.K., Graeff, A.S., Yarchoan, R. and Strober, W. Enterically induced immunological tolerance. I. Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyer's patch after protein feeding. *J. Immunol.* 1981, **126**, 2079
- 49 Müller-Schoop, J.W. and Good, R.A. Functional studies of Peyer's patches: Evidence for their participation in intestinal immune responses. *J. Immunol.* 1975, **114**, 1757
- 50 Coffman, R.L., Lebman, D.A. and Shrader, B. Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J. Exp. Med.* 1989, **170**, 1039
- 51 Sonoda, E., Matsumoto, R., Hitoshi, Y., Ishi, T., Sugimoto, M., Araki, S. *et al.* Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. *J. Exp. Med.* 1989, **170**, 1415
- 52 Beagley, K.W., Eldridge, J.H., Kiyono, H., Everson, M.P., Koopman, W.J., Honjo, T. and McGhee, J.R. Recombinant murine IL-5 induced high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. *J. Immunol.* 1988, **141**, 2035
- 53 Beagley, K.W., Eldridge, J.H., Lee, F., Kiyono, H., Everson, M.P., Koopman, W.J. *et al.* Interleukins and IgA synthesis: human and murine IL-6 induce high rate IgA secretion in IgA-committed B cells. *J. Exp. Med.* 1989, **169**, 2133
- 54 Murray, P.D., McKenzie, D.T., Swain, S.L. and Kagnoff, M.F.
- 55 Coffman, R.L., Shrader, B., Carty, J., Mosmann, T.R. and Bond, M.W. A mouse T cell product that preferentially enhances IgA production. I. Biologic characterization. *J. Immunol.* 1987, **139**, 3685
- 56 Bond, M.W., Shrader, B., Mosmann, T.R. and Coffman, R.L. A mouse T cell product that preferentially enhances IgA production. II. Physicochemical characterization. *J. Immunol.* 1987, **139**, 3691
- 57 Harriman, G.R., Kunitomo, D.Y., Elliott, J.F., Paetkau, V. and Strober, W. The role of IL-5 in IgA B cell differentiation. *J. Immunol.* 1988, **140**, 3033
- 58 Lebman, D.A. and Coffman, R.L. The effects of IL-4 and IL-5 on the IgA response by murine Peyer's patch B cell subpopulations. *J. Immunol.* 1988, **141**, 2050
- 59 Yokota, T., Arai, N., de Vries, J., Spits, H., Banchemereau, J., Zlotnik, A. *et al.* Molecular biology of interleukin 4 and interleukin 5 genes and biology of their products that stimulate B cells, T cells and hemopoietic cells. *Immunol. Rev.* 1988, **102**, 137
- 60 Guy-Grand, D.C., Griscelli, D.C. and Vassalli, P. The mouse gut T lymphocyte, a novel type of T cell. *J. Exp. Med.* 1978, **148**, 1661
- 61 Tagliabue, A., Luini, W., Soldateschi, D. and Boraschi, D. Natural killer activity of gut mucosal lymphoid cells in mice. *Eur. J. Immunol.* 1981, **11**, 919
- 62 McDermott, R.P., Franklin, G.O., Jenkins, K.M., Kodner, I.J., Nash, G.S. and Weinrieb, I.J. Human intestinal mononuclear cells. I. Investigation of antibody-dependent, lectin-induced and spontaneous cell-mediated cytotoxic capabilities. *Gastroenterology* 1980, **78**, 47
- 63 Nauss, K.M., Pavlina, T.M., Kumar, V., and Newbergen, P.M. Functional characteristics of lymphocytes isolated from the rat large intestine. *Gastroenterology* 1984, **86**, 468
- 64 Davies, M.D.J. and Parrott, D.M.W. Cytotoxic T cells in small intestine epithelium, lamina propria and lung lymphocytes. *Immunology* 1981, **44**, 367
- 65 Klein, J.R. and Kagnoff, M.F. Nonspecific recruitment of cytotoxic effector cells in the intestinal mucosa of antigen-primed mice. *J. Exp. Med.* 1984, **160**, 1931
- 66 Ernst, P.B., Clark, D.A., Rosenthal, K.L., Befus, A.D. and Bienenstock, J. Detection and characterization of cytotoxic T lymphocyte precursors in the murine intraepithelial leukocyte population. *J. Immunol.* 1986, **136**, 2121
- 67 Ernst, P.B., Petit, A., Befus, A.D., Clark, D.A., Rosenthal, K.L., Ishizaka, T. and Bienenstock, J. Murine intestinal intraepithelial lymphocytes. II. Comparison of freshly isolated and cultured intraepithelial lymphocytes. *Eur. J. Immunol.* 1984, **14**, 216
- 68 Kagnoff, M.F. and Campbell, S. Functional characteristics of Peyer's patch lymphoid cells. I. Induction of humoral antibody and cell-mediated allograft reactions. *J. Exp. Med.* 1974, **139**, 398
- 69 Kagnoff, M.F. Effects of antigen-feeding on intestinal and systemic immune responses. I. Priming of precursor cytotoxic T cells by antigen feeding. *J. Immunol.* 1978, **120**, 395
- 70 Klein, J.R., Lefrancois, L. and Kagnoff, M.F. A murine cytotoxic T lymphocyte clone from the intestinal mucosa that is antigen-specific for proliferation and displays broadly reactive inducible cytotoxic activity. *J. Immunol.* 1985, **135**, 3697
- 71 Klein, J.R. and Kagnoff, M.F. Spontaneous *in vitro* evolution of lytic specificity of cytotoxic T lymphocyte clones isolated from murine intestinal epithelium. *J. Immunol.* 1987, **138**, 58
- 72 Fujihashi, K., Taguchi, T., McGhee, J.R., Eldridge, J.H., Bruce, M.G., Green, D.R. *et al.* Regulatory function for murine intraepithelial lymphocytes. Two subsets of CD3⁺ T cell receptor-1⁺ intraepithelial lymphocyte T cells abrogate oral tolerance. *J. Immunol.* 1990, **145**, 2010
- 73 Goodman, T. and Lefrancois, L. Expression of the $\gamma\delta$ T-cell receptor on intestinal CD3⁺ intraepithelial lymphocytes. *Nature* 1988, **333**, 855
- 74 Bonneville, M., Janeway, C.A. Jr., Ito, K., Haser, W., Ishida, I., Nakanishi, N. and Tonegawa, S. Intestinal intraepithelial lymphocytes are a distinct set of $\gamma\delta$ T cells. *Nature* 1988, **336**, 479
- 75 Issekutz, T.B. The response of gut-associated T lymphocytes to intestinal viral immunization. *J. Immunol.* 1984, **133**, 2955
- 76 Offit, P.A. and Dudzik, K.I. Rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosal surface after rotavirus infection. *J. Virol.* 1989, **63**, 3507
- 77 Dimmock, N.J. Mechanisms of neutralization of animal viruses. *J. Gen. Virol.* 1984, **65**, 1015
- 78 Taylor, H.P. and Dimmock, N.J. Mechanism of neutralization of influenza-virus by secretory IgA is different from that of monomeric IgA or IgG. *J. Exp. Med.* 1985, **161**, 198

- Rev. 1988, **52**, 296
- 80 Magnusson, K-E and Stjernström, I. Mucosal barrier mechanisms. Interplay between secretory IgA (S-IgA), IgG and mucins on the surface properties and association of salmonellae with intestine and granulocytes. *Immunology* 1982, **45**, 239
- 81 Ogra, P.L., Leibovitz, E.E. and Zhao-Ri, G. Oral immunization and secretory immunity to viruses. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 73
- 82 Gartner, S., Markovits, P., Markovitz, D.M., Kaplan, M., Gallo, R.C. and Popovic, M. The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science* 1986, **233**, 215
- 83 Virelizier, J.L. Host defenses against influenza: The role of antihemagglutinin antibody. *J. Immunol.* 1975, **115**, 434
- 84 Taylor, H.P., Armstrong, S.J. and Dimmock, N.J. Quantitative relationships between an influenza virus and neutralizing antibody. *Virology* 1987, **159**, 288
- 85 Dimmock, N.J., Taylor, J.P. and Carver, A.S. Interaction of neutralized influenza virus with avian and mammalian cells. In: *Segmented Negative Strand Viruses* (Eds Compans, R.W. and Bishop, D.W.L.) Raven Press, New York, 1983, p. 355
- 86 Wold, A.E., Mestecky, J., Tomana, M., Kobata, A., Ohbayashi, H., Endo, T. and Svanborg-Eden, C. Secretory immunoglobulin A carries oligosaccharide receptors for *Escherichia coli* type 1 fimbrial lectin. *Infect. Immun.*, 1990, **58**, 3073
- 87 Abraham, S.N. and Beachey, E.H. Host defenses against adhesion of bacteria to mucosal surfaces. In: *Advances in Host Defense Mechanisms* (Eds Gallin, J.F. and Fauci, A.S.) Vol. 4, Raven Press, New York, 1985, p. 63
- 88 Arnold, R.R., Russell, J.E., Devine, S.M. and Russell, R.B. Antimicrobial activity of the secretory innate defense factors of lactoferrin, lactoperoxidase and lysozyme. In: *Cariology* (Ed. Guggenheim, B.) S. Karger, Basel, Switzerland, 1979, p. 75
- 89 Shedlofsky, S. and Freter, R. Synergism between ecologic and immunologic control mechanisms of intestinal flora. *J. Infect. Dis.* 1974, **129**, 296
- 90 Tagliabue, A. Immune response to oral salmonella vaccines. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 225
- 91 Andre, C., Lambert, R., Bazin, H. and Vaerman, J.-P. Interference of oral immunization with the intestinal absorption of heterologous albumin. *Eur. J. Immunol.* 1974, **4**, 701
- 92 Walker, W.A., Isselbacher, K.J. and Bloch, K.J. Intestinal uptake of macromolecules: Effect of oral immunization. *Science* 1972, **177**, 608
- 93 Cunningham-Rundles, C., Brandeis, W.E., Good, R.A. and Day, N.K. Milk precipitins, circulating immune complexes and IgA deficiency. *Proc. Natl Acad. Sci. USA* 1978, **75**, 3387
- 94 Bloch, K.J. and Walker, W.A. Effect of locally induced intestinal anaphylaxis on the uptake of a bystander antigen. *J. Clin. Immunol.* 1981, **67**, 312
- 95 Tolo, K., Brandtzaeg, P. and Jonsen, J. Mucosal penetration of antigen in the presence or absence of serum-derived antibody. An *in vitro* study of rabbit oral and intestinal mucosa. *Immunology* 1977, **33**, 733
- 96 Russell-Jones, G.J., Ey, P.L. and Reynolds, B.L. Inhibition of cutaneous anaphylaxis and Arthus reactions in the mouse by antigen-specific IgA. *Int. Arch. Allerg. Appl. Immunol.* 1981, **66**, 316
- 97 Russell-Jones, G.J., Ey, P.L. and Reynolds, B.L. The ability of IgA to inhibit the complement-mediated lysis of target red blood cells sensitized with IgG antibody. *Mol. Immunol.* 1980, **17**, 1173
- 98 Elson, C.O. Cholera toxin and its subunits as potential oral adjuvants. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 29
- 99 Elson, C.O. and Ealding, W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.* 1984, **132**, 2736
- 100 Holmgren, J., Clemens, J., Sack, D.A., Sanchez, J. and Svennerholm, A.-M. Oral immunization against cholera. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 197
- 101 Bergmann, K-C and Waldman, R.H. Oral immunization with influenza virus: Experimental and clinical studies. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 83
- 102 Bergmann, K-C, Waldman, R.H., Tischner, H. and Pohl, W.D. Antibody in tears, saliva and nasal secretions following oral immunization of humans with inactivated influenza virus vaccine. *Int. Arch. Allerg. Appl. Immunol.* 1986, **80**, 107
- 103 Waldman, R.H., Stone, J., Bergmann, K-C., Khakoo, R., Lazzell, V., Jacknowitz, A. et al. Secretory antibody following oral influenza immunization. *Am. J. Med. Sci.* 1986, **292**, 367
- 104 Mestecky, J. and McGhee, J.R. New strategies for oral immunization. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 1
- 105 Hemmings, W.A. *Antigen Absorption by the Gut*. University Park Press, Baltimore, MD, 1978, p. 1
- 106 Black, R.E., Levine, M.M., Young, C. et al. Immunogenicity of Ty21a attenuated *Salmonella typhi* given with sodium bicarbonate or in enteric-coated capsules. *Dev. Biol. Stand.* 1983, **53**, 9
- 107 Lycke, N., Lindholm, L. and Holmgren, J. Cholera antibody production *in vitro* by peripheral blood lymphocytes following oral immunization of humans and mice. *Clin. Exp. Immunol.* 1985, **62**, 39
- 108 McKenzie, S.J. and Halsey, J.F. Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. *J. Immunol.* 1984, **133**, 1818
- 109 Betley, M., Miller, V. and Mekalanos, J. Genetics of bacterial enterotoxins. *Annu. Rev. Microbiol.* 1986, **40**, 577
- 110 Cuatrecasas, P. Gangliosides and membrane receptors for cholera toxin. *Biochemistry* 1973, **12**, 3558
- 111 Pierce, N. and Gowans, J. Cellular kinetics of the intestinal immune response to cholera toxin in rats. *J. Exp. Med.* 1975, **142**, 1550
- 112 Elson, C.O. and Ealding, W. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. *J. Immunol.* 1984, **133**, 2892
- 113 Elson, C.O. and Ealding, W. Genetic control of the murine immune response to cholera toxin. *J. Immunol.* 1985, **135**, 930
- 114 Elson, C.O. and Ealding, W. Ir gene control of the murine secretory IgA response to cholera toxin. *Eur. J. Immunol.* 1987, **17**, 425
- 115 Pierce, N.F. and Cray, W.C. Determinants of the localization, magnitude and duration of a specific IgA plasma cell response in enterically immunized rats. *J. Immunol.* 1982, **128**, 1311
- 116 Lycke, N. and Holmgren, J. Intestinal mucosal memory and presence of memory cells in lamina propria and Peyer's patches in mice 2 years after oral immunization with cholera toxin. *Scand. J. Immunol.* 1986, **23**, 611
- 117 McKenzie, S. and Halsey, J. Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. *J. Immunol.* 1984, **133**, 1818
- 118 Bessen, D. and Fischetti, V. Influence of intranasal immunization with synthetic peptides corresponding to conserved epitopes of M protein on mucosal colonization by group A streptococci. *Infect. Immun.* 1988, **56**, 2666
- 119 Tamura, S., Samegai, Y., Kurata, H., Nagamine, T., Aizawa, C. and Kurata, T. Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. *Vaccine* 1988, **6**, 409
- 120 Liang, X., Lamm, M. and Nedrud, J. Oral administration of cholera toxin-sendai virus conjugates potentiates gut and respiratory immunity against sendai virus. *J. Immunol.* 1988, **141**, 1495
- 121 Czerkinsky, C., Russell, M.W., Lycke, N., Lindblad, M. and Holmgren, J. Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosa tissues. *Infect. Immun.* 1989, **57**, 1072
- 122 Clements, J.D., Hartzog, N.M. and Lyon, F.L. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. *Vaccine* 1988, **6**, 269
- 123 Chen, K.S. and Quinnan, G.V. Efficacy of inactivated influenza vaccine delivered by oral administration. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 101
- 124 Nedrud, J.G., Liang, X., Hague, N. and Lamm, M.E. Combined oral/nasal immunization protects mice from Sendai virus infection. *J. Immunol.* 1987, **139**, 3484
- 125 Liang, X., Lamm, M.E. and Nedrud, J.G. Cholera toxin as a mucosal adjuvant for respiratory antibody responses in mice. *Reg. Immunol.* 1989, **2**, 244
- 126 Elson, C.O. Cholera toxin as a mucosal adjuvant—the effect of H-2 genes. *Fed. Proc.* 1987, **46**, 1778
- 127 Cryz, S.J., Cross, A.S., Sadoff, J.C. and Fürer, E. Synthesis and characterization of *Escherichia coli* O18 O-polysaccharide conjugate vaccines. *Infect. Immun.* 1990, **58**, 373
- 128 Szu, S.C., Li, X., Schneerson, R., Vickers, J.H., Bryla, D. and Robbins, J.B. Comparative immunogenicities of Vi polysaccharide-protein conjugates composed of a cholera toxin or its B subunit as a carrier bound to high- or lower-molecular weight Vi. *Infect. Immun.* 1989, **57**, 3823
- 129 Que, J.U., Cryz, S.J., Ballou, R., Fürer, E., Gross, M., Young, J. et al. Effect of carrier selection on immunogenicity of protein conjugate vaccines against *Plasmodium falciparum* circumsporozoites. *Infect. Immun.* 1988, **56**, 2645
- 130 Lycke, N. and Holmgren, J. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology* 1986, **59**, 301
- 131 Robbins, J., Schneerson, R., Szu, S.C., Fattom, A., Yang, Y., Lagergard, T. et al. Prevention of invasive bacterial diseases by immunization with polysaccharide-protein conjugates. *Curr. Top.*

- Microbiol. Immunol.* 1989, **146**, 169
- 132 Verheul, A.F.M., Verstees, A.A., DeReuver, M.J., Jansze, M. and Snippe, H. Modulation of the immune response to pneumococcal type 14 capsular polysaccharide-protein conjugates by the adjuvant Quil A depends on the properties of the conjugates. *Infect. Immun.* 1989, **57**, 1078
 - 133 Eldridge, J.H., Gilley, R.M., Staas, J.K., Moldoveanu, Z., Meulbroek, J.A. and Tice, T.R. Biodegradable microspheres: Vaccine delivery system for oral immunization. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 59
 - 134 Moldoveanu, Z., Staas, J.K., Gilley, R.M., Ray, R., Compans, R.W., Eldridge, J.H. *et al.* Immune responses to influenza virus in orally and systemically immunized mice. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 91
 - 135 Beck, L.R., Ramas, R.A., Flowers, C.E. *et al.* Clinical evaluation of injectable biodegradable contraceptive system. *Am. J. Obstet. Gynecol.* 1981, **140**, 799
 - 136 Beck, L.R., Pope, V.Z., Cowsar, D.R., Lewis, D.H. and Tice, T.R. Evaluation of a new three-month injectable contraceptive microsphere system in primates. *Contracep. Del. Syst.* 1980, **1**, 79
 - 137 Beck, L.R., Pope, V.Z., Flowers, C.E., Cowsar, D.R., Tice, T.R., Dunn, R.L. *et al.* Poly (DL-lactide-co-glycolide)/nortisterone microcapsules: An injectable biodegradable contraceptive. *Biol. Reprod.* 1983, **28**, 186
 - 138 Miller, R.A., Brady, J.M. and Cutright, D.W. Degradation rates of oral resorbable implants (polylactates and polyglycolates): Rate modification with changes in PLA/PGA copolymer ratios. *J. Biomed. Mat. Res.* 1977, **11**, 711
 - 139 Eldridge, J.H., Staas, J.K., Meulbroek, J.A., McGhee, J.R., Tice, T.R. and Gilley, R.M. Biodegradable microspheres as a vaccine delivery system. *Mol. Immunol.* 1991, **28**, 287
 - 140 Eldridge, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.R. and Gilley, R.M. Biodegradable and biocompatible poly(DL-lactide-Co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. *Infect. Immun.* 1991, **59**, 2978
 - 141 Eldridge, J.H., Hammond, C.J., Meulbroek, J.A., Staas, J.K., Gilley, R.M. and Tice, T.R. Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches. *J. Controlled Release* 1990, **11**, 205
 - 142 Eldridge, J.H., Meulbroek, J.A., Staas, J.K., Tice, T.R. and Gilley, R.M. Vaccine-containing biodegradable microspheres specifically enter the gut-associated lymphoid tissue following oral administration and induce a disseminated mucosal immune response. In: *Immunobiology of Proteins and Peptides* (Ed. Atassi, V.M.Z.) Plenum Press, New York, NY, 1989, p. 191
 - 143 O'Hagan, D.T., Rahman, D., McGee, J.P., Jeffery, H., Davies, M.C., Williams, P., Davis, S.S. and Challacombe, S.J. Biodegradable microparticles as controlled release antigen delivery systems. *Immunology* 1991, **73**, 239
 - 144 Curtiss, R. III, Kelly, S.M., Gulig, P.A. and Nakayama, K. Selective delivery of antigens by recombinant bacteria. *Curr. Top. Microbiol. Immunol.* 1989, **146**, 35
 - 145 Curtiss, R., Goldschmitt, R., Pastian, R., Lyons, M., Michalek, S.M. and Mestecky, J. Cloning virulence determinants from *Streptococcus mutans* and the use of recombinant clones to construct bivalent oral vaccine strains to confer protective immunity against *S. mutans*-induced dental caries. In *Molecular Microbiology and Immunobiology of Streptococcus mutans* (Eds Hamada, S., Michalek, S.M., Kiyono, H. *et al.*) Elsevier, Amsterdam, 1986, p. 173
 - 146 Poirier, T.P., Keho, M.A. and Beachey, E.H. Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. *J. Exp. Med.* 1988, **168**, 25
 - 147 Sjostedt, A., Sandstrom, G. and Tarnvik, A. Immunization of mice with an attenuated *Salmonella typhimurium* strain expressing a membrane protein of *Francisella tularensis*. A model for identification of bacterial determinants relevant to the host defence against tularemia. *Res. Microbiol.* 1990, **141**, 887
 - 148 Schodel, F. and Will, H. Expression of hepatitis-B virus antigens in attenuated *Salmonella* for oral immunization. *Res. Microbiol.* 1990, **141**, 831
 - 149 Cohen, S., Powell, C.J., Dubois, D.R., Hartman, A., Summers, P.L. and Eckels, K.H. Expression of the envelope antigens of dengue virus in vaccine strains of *Salmonella*. *Res. Microbiol.* 1990, **141**, 855
 - 150 Bowen, J.C., Alpar, O., Philpotts, R., Roberts, I.S. and Brown, M.R.W. Preliminary studies on infection by attenuated *Salmonella* in guinea pig and on expression of herpes simplex virus. *Res. Microbiol.* 1990, **141**, 873
 - 151 Salasvidal, E., Plebanski, M., Castro, S., Perales, G., Mata, E., Lopez, S. and Arias, C.F. Synthesis of the surface glycoprotein of rotavirus SA11 in the *aroA* strain of *Salmonella typhimurium* SL3261. *Res. Microbiol.* 1990, **141**, 883
 - 152 Hale, T.L. Hybrid vaccines using *Escherichia coli* as an antigen carrier. *Res. Microbiol.* 1991, **141**, 913
 - 153 Sory, M.P. and Cornelis, G.R. Delivery of the cholera toxin-B subunit by using a recombinant *Yersinia enterocolitica* strain as a live oral carrier. *Res. Microbiol.* 1990, **141**, 921
 - 154 Barletta, R.G., Snapper, B., Cirillo, J.D., Connell, N.D., Kim, D.D., Jacobs, W.R. and Bloom, B.R. Recombinant BCG as a candidate oral vaccine vector. *Res. Microbiol.* 1990, **141**, 931
 - 155 Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T. *et al.* New use of BCG for recombinant vaccines. *Nature*, **351**, 456
 - 156 Gerritse, K., Posno, M., Schellekens, M.M., Boersma, W.J.A. and Classen, E. Oral administration of TNP-*Lactobacillus* conjugates in mice. A model for evaluation of mucosal and systemic immune responses and memory formation elicited by transformed *Lactobacilli*. *Res. Microbiol.* 1990, **141**, 955

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Detection of low-density cell-surface molecules using biotinylated fluorescent microspheres

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Biotinylated fluorescent microspheres have been developed as a reagent for studying antigens and receptors expressed at the cell surface. Labeling of antigen or receptor was accomplished by crosslinking biotinylated microspheres through streptavidin to corresponding biotinylated antibodies or ligands. Detection of labeled cells by flow microfluorimetry provided an extremely sensitive means for the analysis and potential manipulation of heterogeneous cell populations. The data indicate that cells bearing fewer than 200 surface antigen-antibody complexes per cell are readily detectable by this approach. Crosslinked to a selected biotinylated peptide immunogen, biotinylated fluorescent microspheres also allowed the labeling and detection of hybridoma cells bearing antigen-specific surface immunoglobulin.

Introduction

The use of fluorescent immunoreagents in combination with flow microfluorimetry to identify, quantify and isolate cells according to their surface antigens and receptors permits the monitoring and manipulation of heterogeneous cell populations at the single-cell level. Hence, flow microfluorimetry [1,2] has been extremely useful in studies of cell differentiation [3,4], plasma membrane receptors [5-7], and genes coding for antigens expressed at the cell surface, including their chromosomal mapping and cloning by expression [8-10]. Due to the limited fluorescence of conventional soluble immunoreagents, however, cells bearing very low densities of antigen or receptor of interest typically

escape detection. While it has been estimated by extrapolation that flow microfluorimeters possessing newly developed optics and lasers of high power are capable of detecting cells bearing as few as 1500 fluorescein molecules [11], significantly greater intensities of cell-associated fluorescence are required for conventional microfluorimetry. Previous studies have demonstrated that the detection of rare cells within heterogeneous populations may be facilitated by attaching appropriate immunoreagents to highly fluorescent latex microspheres [12,13]. In order to detect rare cells which, in addition, bear antigens or receptors at exceedingly low densities we now have developed biotinylated fluorescent microspheres as a means for selectively labeling cell surfaces. We have found that biotinylated microspheres used in combination with streptavidin and biotinylated antibodies permit the labeling of cells bearing fewer than 200 antigen-bound IgG molecules, thus extending the sensitivity of flow microfluorimetric detection of

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Abbreviations: SPDP, *N*-succinimidyl-3-(pyridyldithio)-propionate; NHS-biotin, *N*-hydroxysuccinimidobiotin.

low-density cell-surface antigens by one to two orders of magnitude.

Materials and Methods

Albumin-conjugated erythrocytes. Bovine serum albumin was attached to surface sulfhydryl groups of sheep erythrocytes using *N*-succinimidyl-3-(pyridyldithio)propionate (SPDP) [14]. Albumin (10 mg/ml in 0.1 M NaCl/0.07 M Na₂HPO₄·7H₂O/0.03 M NaH₂PO₄·H₂O (pH 7.5)) was incubated with SPDP (6.5 mg/ml) for 30 min at 23°C and the thiolated product was isolated by dialysis at 4°C against phosphate-buffered saline (2.7 mM KCl/0.9 mM CaCl₂/0.5 mM MgCl₂·6H₂O/137 mM NaCl/1.4 mM KH₂PO₄/8.1 mM Na₂HPO₄ (pH 7.4)). Erythrocytes (1.25 ml of a 2% suspension) were incubated with dithiothreitol at 35 mM in phosphate-buffered saline for 1 h at 23°C. 200 µl washed, packed cells were then suspended in 0.2 ml phosphate-buffered saline and incubated overnight with 0.2 ml thiolated albumin. The resulting albumin-conjugated erythrocytes were stored for no longer than 1 week in phosphate-buffered saline at 4°C, and were washed prior to use.

Biotinylated anti-albumin IgG. Antibodies to bovine serum albumin were purified from sera of rabbits previously immunized with albumin in the form of haptenic carrier [15]. Purification of antibodies was accomplished by (NH₄)₂SO₄ precipitation (50% saturated solution), DEAE-cellulose chromatography [16], and affinity chromatography on immobilized bovine serum albumin (Affi-Gel-10, Bio-Rad) using 4 M guanidine-HCl/50 mM Tris-HCl (pH 7.0) as elution buffer. Biotin was covalently attached to anti-albumin IgG using *N*-hydroxysuccinimidobiotin (NHS-biotin) [17] with IgG and NHS-biotin at final concentrations of $1.6 \cdot 10^{-6}$ M and $8.3 \cdot 10^{-5}$ M, respectively. As determined using an enzyme-linked assay [18] biotinylated anti-albumin IgG retained essentially all of its antigen binding activity, and was precipitated specifically (82%) by avidin [19].

Measurements of biotinylated anti-albumin IgG-albumin complexes or erythrocytes using ¹²⁵I-labeled protein A. Albumin-conjugated erythrocytes were incubated at $5 \cdot 10^7$ cells/ml with specified dilutions of biotinylated anti-albumin IgG in phos-

phate-buffered saline/1% normal rabbit serum, for 12 h at 4°C. Following three washes in 10 ml phosphate-buffered saline/0.5% gelatin, $5 \cdot 10^7$ erythrocytes were incubated for 1 h at 23°C in a total volume of 0.5 ml phosphate-buffered saline with ¹²⁵I-labeled protein A (1.19 µCi/µg) (New England Nuclear) at increasing concentrations. The number of molecules of ¹²⁵I-labeled protein A specifically bound per cell was determined and served as an estimate of the cell-surface density of anti-albumin IgG-albumin complexes.

Synthesis of biotinylated fluorescent microspheres. Biotinylated fluorescent microspheres were prepared by reacting 0.15 ml of washed, sonicated amino-microspheres (Covalent Technologies, green FX spheres, 1 µm diameter) in 0.75 ml of 0.1 M NaCl/0.07 M Na₂HPO₄·7H₂O/0.03 M NaH₂PO₄·H₂O (pH 7.5), with NHS-biotin (0.3 mg in 0.75 ml 25% dimethylformamide in water) for 1 h at 23°C. Washed biotinylated microspheres were stored at 4°C in 1 ml of phosphate-buffered saline/10 mM NaN₃, and were sonicated prior to use.

Biotinylated fluorescent microsphere labeling of albumin-conjugated erythrocytes. Biotinylated fluorescent microsphere labeling of albumin-conjugated erythrocytes was accomplished by first incubating cells ($5 \cdot 10^7$ cells/ml) with specified dilutions of biotinylated anti-albumin IgG for 12 h at 4°C. Cells then were washed three times in 10 ml phosphate-buffered saline/1% normal rabbit serum, incubated at $5 \cdot 10^7$ cells/ml with streptavidin (5 µg/ml) for 2 h at 23°C, and washed again. Approx. $5 \cdot 10^6$ of these cells were admixed with $1 \cdot 10^8$ biotinylated fluorescent microspheres in 96-well tissue culture plates (Costar No. 3596) (total volume, 0.3 ml phosphate-buffered saline/1% normal rabbit serum), sedimented at $250 \times g$ for 10 min at 4°C, and incubated at 4°C for an additional 30 min. Microspheres and cells were transferred with 0.3 ml phosphate-buffered saline/1% normal rabbit serum to 15 ml conical tubes, underlayered with 1.5 ml of Percoll at 41% in phosphate-buffered saline, and centrifuged at $350 \times g$ for 10 min at 4°C. Microspheres at the Percoll interface were removed. Pelleted cells were resuspended in 0.5 ml phosphate buffered saline/1% normal rabbit serum, and were analyzed by flow microfluorimetry (Ortho Cytofluorograph 50 H,

150 comptuer; 5.13 fluorescence gain, linear scale; 20000 cells per analysis). Forward-angle light-scatter was adjusted to allow for discrimination between labeled erythrocytes and unbound microspheres on the basis of size (2.0 gain, linear scale).

Biotinylated fluorescent microsphere labeling of Y5B12 hybridoma cells. Labeling of Y5B12 cells at surface immunoglobulin was accomplished using preassembled biotinylated microsphere-streptavidin-biotinylated peptide immunogen complexes. Biotinylated fluorescent microspheres (0.25 ml) were incubated for 1 h at 37°C with streptavidin (0.01 ml, 1 mg/ml in phosphate-buffered saline/1% bovine serum albumin), washed, resuspended, and sonicated in phosphate-buffered saline/1% bovine serum albumin. Biotinylated peptide then was added (0.05 ml, 0.5 mg/ml) and was incubated with microspheres for 1 h at 37°C. The resulting peptide-coated microspheres were washed

and sonicated prior to use. Biotinylated peptide was prepared by incubating peptide (0.25 ml, 1 mg/ml) with NHS-biotin (0.25 ml, 1 mg/ml) for 2 h at 30°C in 0.1 M sodium carbonate-bicarbonate/12.5% dimethylformamide (pH 10.0) and isolating the biotinylated product by chromatography on Sephadex G-15. Flow microfluorimetry and the incubation of cells with microspheres were performed as described above.

Results and Discussion

In testing biotinylated fluorescent microspheres and in assessing their sensitivity, we selected a model system in which bovine serum albumin covalently bound to sheep erythrocytes using SPDP [14] served as a targeted surface antigen. Labeling of surface antigen with microspheres was accomplished in two steps. First, albumin-con-

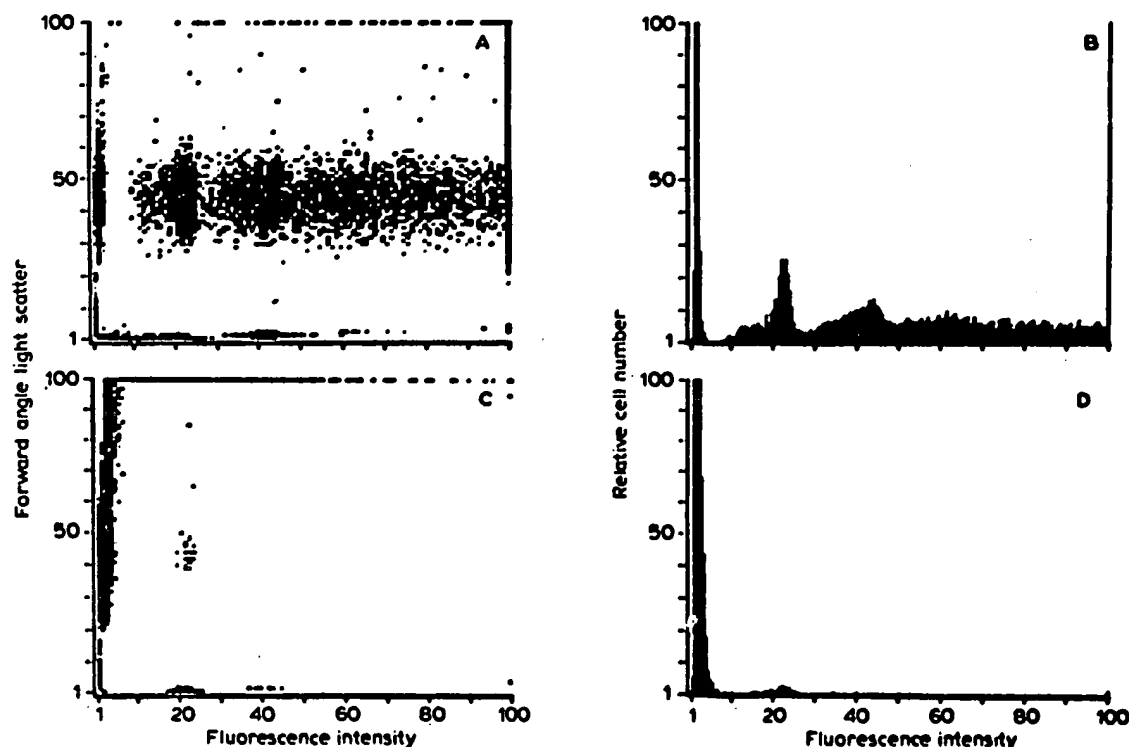


Fig. 1. Labeling of albumin-conjugated erythrocytes with anti-albumin IgG and biotinylated fluorescent microspheres. Following sequential incubations with biotinylated anti-BSA IgG (1:50 dilution), streptavidin and biotinylated microspheres, greater than 90% of albumin-conjugated erythrocytes were fluorescently labeled as measured by flow microfluorimetry (panels A and B). As assessed by the co-incubation of cells with biotinylated fluorescent microspheres and excess (+)-biotin (0.05 mg/ml) (panels C and D), nonspecific binding of microspheres to cells was less than 1% of total.

jugated erythrocytes were incubated with affinity-purified biotinylated antibodies to albumin. Biotinylated fluorescent microspheres then were crosslinked through streptavidin to biotinylated antibody-albumin complexes at the cell surface. In initial experiments using antibody at a dilution of 1:50 ($2.5 \cdot 10^{-8}$ M IgG), greater than 90% of these cells were labeled, with 10% bearing greater than five microspheres per cell (Fig. 1, panels A and B). As assessed by the co-incubation of cells either with biotinylated microspheres and (+)-biotin (0.1 mg/ml) (Fig. 1, panels C and D) or with biotinylated anti-albumin IgG and bovine serum albumin (0.1 mg/ml) (not shown), non-specific binding of biotinylated fluorescent microspheres to cells was less than 1% of total.

By the following means, use of the albumin-conjugated erythrocyte model antigen system provided an estimate of the lower limit of the density of cell-surface antigen detectable by biotinylated fluorescent microsphere labeling. Erythrocytes bearing albumin were first incubated with specified decreasing concentrations of biotinylated anti-albumin IgG. The average number of anti-

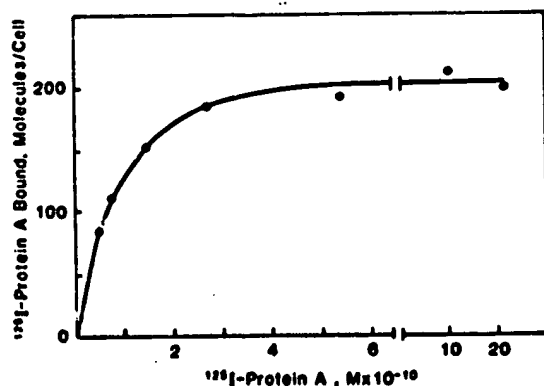


Fig. 2. Binding of ^{125}I -labeled protein A to IgG-albumin complexes on erythrocytes. Erythrocytes bearing albumin were incubated with biotinylated anti-albumin IgG at a dilution of 1:100, washed, and incubated with increasing concentrations of ^{125}I -labeled protein A. The mean number of molecules of ^{125}I -labeled protein A specifically bound per erythrocyte (approx. 200) served as a direct estimate of the mean density of IgG-albumin complexes formed using this dilution of antibody. At all concentrations, nonspecific binding of ^{125}I -labeled protein A was less than 3% of total as determined following the co-incubation of erythrocytes with antibody and excess bovine serum albumin (0.1 mg/ml).

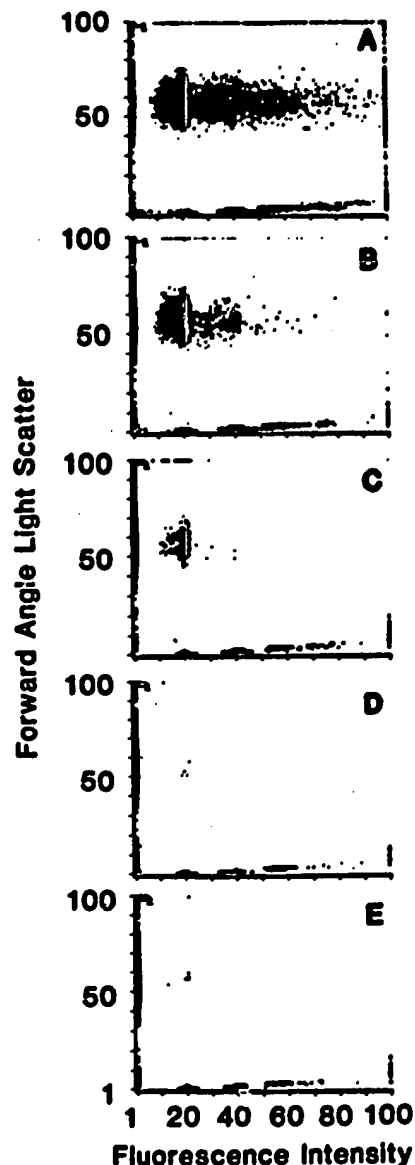


Fig. 3. Biotinylated fluorescent microsphere labeling of erythrocytes bearing low densities of biotinylated anti-albumin IgG-albumin complexes. Dilutions of biotinylated anti-albumin IgG used in albumin-conjugated erythrocyte labeling were panel A, 1:100; B, 1:500; D, 1:100; E, 1:100. The molar concentrations of antibody corresponding to these dilutions are 1:100, $1.25 \cdot 10^{-8}$ M; 1:500, $2.5 \cdot 10^{-9}$ M; 1:1500, $8.3 \cdot 10^{-10}$ M. Controls for nonspecific binding were panel D, cells incubated with biotinylated anti-albumin IgG in the presence of excess albumin (0.1 mg/ml); panel E, cells incubated with biotinylated fluorescent microspheres in the presence of excess (+)-biotin (0.05 mg/ml).

body-albumin complexes formed per cell then was measured directly using ^{125}I -labeled protein A of known specific radioactivity and Fc-region binding activity (Fig. 2). Assuming monovalency of IgG for protein A, incubation with antibody at a dilution of 1:100 yielded cells bearing approx. 200 IgG-albumin complexes. Densities of IgG-albumin complexes resulting from the incubation of cells with antibody at dilutions of 1:500 or 1:1500 were too low for detection by the ^{125}I -labeled protein A binding assay. Therefore, these densities were estimated both by extrapolation, and by a more conservative bimodal model (see below). When labeled using streptavidin and biotinylated fluorescent microspheres, those populations of albumin-conjugated erythrocytes incubated with biotinylated albumin antibodies at dilutions of 1:100, 1:500, and 1:1500 were shown by flow microfluorimetry to contain labeled cells at frequencies of 51.7%, 23.2% and 7%, respectively (Fig. 3, panels A-C). Binding of antibody and of biotinylated microspheres to cells was specific: the co-incubation of excess albumin with antibody and cells (Fig. 3, panel D), or of excess (+)biotin with microspheres and cells (Fig. 3E), reduced cell-associated fluorescence to background levels.

From these data, two estimates of the density of surface antigens detectable with biotinylated fluorescent microspheres can be made according to two alternative assumptions. These estimates are necessary, since the direct measurement of IgG-albumin complexes using ^{125}I -labeled protein A was not feasible at densities below 200 IgG molecules per cell. In the first instance, it is assumed that the mean density of IgG-albumin complexes occurring on erythrocytes decreases proportionally with the dilution of antibody employed. Incubation of cells with biotinylated anti-albumin IgG at a dilution of 1:100 resulted in approximately 200 IgG-albumin complexes per cell (Fig. 2). Accordingly, incubations with antibody at dilutions of 1:500 and 1:1500 are predicted to yield cells possessing mean densities of 40 and 13 IgG-albumin complexes per cell, respectively. Flow microfluorimetry using biotinylated fluorescent microspheres detected some of the cells incubated with antibody at each of these dilutions (Fig. 3). However, it is possible that in each cell population, those cells labeled with microspheres possessed above-average densities of

immune complexes. A second estimate of the sensitivity of biotinylated fluorescent microspheres which accounts for this possibility can be made through an alternative assumption. As stated, the mean density of IgG-albumin complexes on cells incubated with antibody at a dilution of 1:100 was measured directly at 200 per cell. Approx. 51% of these cells were labeled by biotinylated microspheres. By the most conservative interpretation in which only fluorescent cells are assumed to bear biotinylated anti-albumin IgG-albumin complexes and nonfluorescent cells are assumed to bear no biotinylated anti-albumin IgG-albumin complexes, the maximal mean density of IgG-albumin complexes for the fluorescent subpopulation is calculated at $200/0.517$, i.e., 387 per cell. Therefore it is estimated that 100% of those cells bearing an average of 387 complexes per cell were detected. Lack of additional information concerning the distribution of the density of IgG-albumin complexes among cells precludes the assignment of a more precise limit for the sensitivity of biotinylated fluorescent microspheres in cell surface antigen detection. However, the above analyses of available data indicate that cells bearing approx. 200 antigen molecules, and perhaps less, are detected by this technique.

Several additional features of the biotinylated fluorescent microsphere assay for cell surface molecules merit discussion. The high sensitivity of these microspheres is attributable, in part, to their high capacity for the attachment of biotinylated reagents. Studies using ^{125}I -labeled streptavidin and [^3H]biotin (not shown) indicated that approx. 10^4 molecules of a selected biotinylated probe can be specifically crosslinked to each microsphere. This high probe density maximizes the probability of a sphere binding to a low-density cell surface molecule. The biotinylated probe may correspond to any of a variety of biotinylated reagents including primary or secondary antibodies, protein A, or receptor ligands. For example, immunogen covalently coupled to fluorescent microspheres previously has been demonstrated to provide sufficient sensitivity for the detection of hybridoma surface immunoglobulins not detectable using soluble fluorescent immunogen and flow microfluorimetry [13]. Using biotinylated fluorescent microspheres in combination with biotinylated immunogen, we

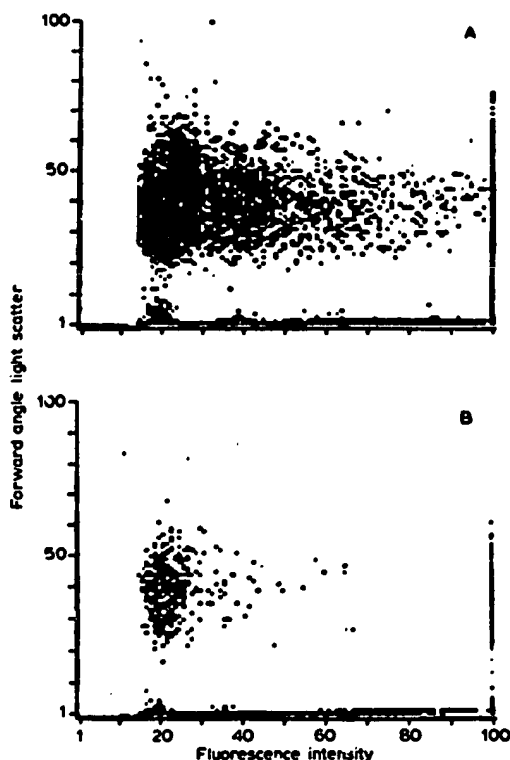


Fig. 4. Labeling of surface immunoglobulin of Y5B12 hybridoma cells using biotinylated peptide immunogen cross-linked to biotinylated fluorescent microspheres. Panel A: flow microfluorimetric analysis of Y5B12 cells labeled with pre-assembled biotinylated peptide immunogen-streptavidin-biotinylated microspheres. Panel B: labeling of Y5B12 cells in the presence of excess underivatized peptide (0.01 mg/ml). Autofluorescence due to unlabeled cells was gated.

have labeled surface immunoglobulin of the cell line Y5B12 (Fig. 4). Y5B12 cells are hybridomas derived from a fusion between P3X-63Ag8653 murine myeloma cells and splenocytes isolated from CB6F₁ mouse immunized with a synthetic peptide [15] corresponding to the putative 26 amino acids of the amino-terminus of the mammalian glycoprotein hormone, erythropoietin. These cells have been shown to secrete IgG₁ antibodies to peptide (unpublished data). In the present analysis, fluorescent microspheres were preassembled as biotinylated microsphere-streptavidin-biotinylated peptide complexes prior to incubation with cells (see Materials and Methods). In flow microfluorimetry, 34% of Y5B12 cells bound peptide-conjugated microspheres, with 23% binding two or more microspheres per cell (Fig. 4A). Levels of nonspecific binding were low, with fewer than 5% of either irrelevant cells (e.g., P3X-63Ag8653 cells, not shown) or Y5B12 cells binding single microspheres in the presence of underivatized peptide (Fig. 4B). Y5B12 cells labeled using biotinylated peptide and fluorescein-labeled streptavidin could not be detected by flow microfluorimetry.

In flow microfluorimetric analyses, the binding of each microsphere to cells increased the intensity of cell-associated fluorescence by discrete intervals (Figs. 1, 3 and 4). Consequently, labeling with biotinylated fluorescent microspheres should improve the resolution and isolation of subpopulations of cells possessing relatively small differences in antigen or receptor densities. When labeled with conventional soluble fluorescent reagents, such subpopulations typically display only minor differences within a continuous spectrum of fluorescence intensities [8-10]. While avidin-conjugated fluorescent microspheres have been prepared previously by a more complex method [20], neither the effects of inherent multiple chemical modifications of avidin on its affinity and specificity for biotin binding nor the limits of sensitivity of these acetylated-avidin microspheres in detecting cell surface antigens have been assessed. In contrast, biotinylated fluorescent microspheres are synthesized through a single reaction between fluorescent aminomicrospheres and *N*-hydroxysuccinimido-biotin, and are coupled directly to unmodified streptavidin. Given their flexible design and demonstrated sensitivity in detecting cell-surface molecules, biotinylated fluorescent microspheres and their derivatives should find wide application in research and clinical laboratories. The generalized approach towards attaching active biotinylated reagents to polymeric microspheres also should be of value in the further development of microspheres used in cell separation [21] and in drug delivery systems [22].

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References

- 1 Shapiro, H.M. (1983) *Cytometry* 3, 227-243
- 2 Cambier, J.C. and Monroe, J.G. (1983) *Methods Enzymol.* 103, 227-245
- 3 Loken, M.R. and Stall, A.M. (1982) *J. Immunol. Methods* 50, R85-R112
- 4 Voet, L., Krefft, M., Mairhofer, H. and Williams, K.L. (1984) *Cytometry* 5, 26-33
- 5 Bohn, B. (1980) *Mol. Cell. Endocrinol.* 20, 1-15
- 6 Murphy, R.F., Powers, S., Cantor, C.R. and Pollack, R. (1984) *Cytometry* 5, 275-280
- 7 Newman, R., Domingo, D., Trotter, J. and Trowbridge, I. (1983) *Nature* 304, 643-646
- 8 Kamarck, M.E., Barbosa, J.A., Kuhn, L., Messer-Peters, P.G., Shulman, L. and Ruddie, F.H. (1983) *Cytometry* 4, 99-108
- 9 Stanners, C.P., Lam, T., Chamberlain, J.W., Stewart, S.S. and Price, G.B. (1981) *Cell* 27, 211-221
- 10 Kavathas, P., Sukhatme, V.P., Herzenberg, L.A. and Parnes, J.R. (1984) *Proc. Natl. Acad. Sci. USA* 81, 7688-7690
- 11 Hoffman, R.A., Burger, D.E. and Brown, M.C. (1984) Poster presented at the Tenth Conference of Analytical Cytology, June 3, 1984, Pacific Grove, CA. Publication available through Ortho Diagnostics Systems, Inc., Westwood, MA, U.S.A.
- 12 Cupp, J.E., Leary, J.F., Cernichiari, E., Wood, J.C.S. and Doherty, R.A. (1984) *Cytometry* 5, 138-144
- 13 Parks, D.R., Bryan, V.M., Oi, V.T. and Herzenberg, L.A. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1962-1966
- 14 Jou, Y.-H., and Bankert, R.B. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2493-2496
- 15 Sue, J.M. and Sytkowski, A.J. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3651-3655
- 16 Goding, J.W. (1983) *Monoclonal Antibodies: Principles and Practice*, pp. 114-115, Academic Press, New York
- 17 Wagener, C., Clark, B.R., Rickard, K.J. and Shively, J.E. (1983) *J. Immunol.* 130, 2302-2307
- 18 Douillard, J.Y. and Hoffman, T. (1983) *Methods Enzymol.* 92, 169-174
- 19 Clark, B.R. and Todd, C.W. (1982) *Anal. Biochem.* 121, 257-262
- 20 Kaplan, M.R., Calef, E., Bercovici, T. and Gitler, C. (1983) *Biochim. Biophys. Acta* 728, 112-120
- 21 Molday, R.S. (1984) in *Cell Separation, Methods and Selected Applications* (Pretlow, T.G. and Pretlow, T.P., eds.), pp. 237-263, Academic Press, New York
- 22 Tritton, T.R. and Wingard, L.B. (1984) in *Microspheres and Drug Therapy* (Illum, D.L., McVie, J.G., Tomlinson, E., eds.), pp. 129-138, Elsevier, Amsterdam

STIC-ILL

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Construction and characterization of recombinant *Vibrio cholerae* strains
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Hase C.C.; Thai L.S.; Boesman-Finkelstein M.; Mar V.L.; Burnette W.N.;
Kaslow H.R.; Stevens L.A.; Moss J.; Finkelstein R.A.

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Hase C C; Thai L S; Boesman-Finkelstein M; Mar V L; Burnette W N; Kaslow
H R; Stevens L A; Moss J; Finkelstein R A
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Construction and Characterization of Recombinant *Vibrio cholerae* Strains Producing Inactive Cholera Toxin Analogs

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The catalytic A subunit of cholera toxin (CT-A) is capable of ADP-ribosylating the guanine nucleotide-binding protein, which regulates cell adenyl cyclase, leading to the life-threatening diarrhea of cholera. Amino acids involved in the enzymatic activity of CT-A have previously been identified. By means of site-directed mutagenesis, an analog of the CT-A subunit gene was created with codon substitutions for both Arg-7 and Glu-112, each of which has been shown to produce subunits lacking ADP-ribosyltransferase activity. The mutated gene fragment was exchanged for the wild-type copy in the previously cloned *ctxAB* operon from El Tor biotype, Ogawa serotype *Vibrio cholerae* strain 3083, which produces CT-2. Further, the zonula occludens toxin gene, *zot*, was inactivated by an insertional mutation to create the new plasmid construct pCT-2*. Additionally, a DNA fragment encoding the B subunit of CT-1 (CT produced by classical biotype, Inaba serotype *V. cholerae* strain 569B) was exchanged for the homologous part in pCT-2*, resulting in the creation of pCT-1*. These plasmid constructs were introduced into the CT-negative *V. cholerae* mutant strain JBK70 (El Tor biotype, Inaba serotype); CT-A⁻B⁺ derivatives CVD101 and CVD103 of classical biotype Ogawa and Inaba serotype strains 395 and 569B, respectively; El Tor biotype Inaba and Ogawa serotype strains C6706 and C7258, respectively, recently isolated in Peru; and O139 (synonym Bengal) strain SG25-1 from the current epidemic in India. Recombinant toxins (CT-1* and CT-2*), partially purified from culture supernatants of transformed JBK70, were shown to be inactive on mouse Y1 adrenal tumor cells and in an in vitro ADP-ribosyltransferase assay. CT-1* and CT-2* reacted with polyclonal and monoclonal antibodies against both A and B subunits of CT. The toxin analogs reacted with antibodies against CT-A and CT-B on cellulose acetate strips and in a G_{M1} enzyme-linked immunosorbent assay; they reacted appropriately with B-subunit epitope-specific monoclonal antibodies in checkerboard immunoblots, and they formed precipitin bands with G_{M1}-ganglioside in Ouchterlony tests. However, the reactions of the modified proteins with anti-A-subunit monoclonal antibodies were weaker than the reactions with wild-type holotoxins. *V. cholerae* strains carrying *ctxA**, with either *ctxB-1* or *ctxB-2*, and inactivated *zot* genes were created by homologous recombination. The recombinant strains and the purified toxin analogs were inactive in the infant rabbit animal model. These strains may have use as attenuated live vaccines; the analog toxins themselves might have important applications in conjugate vaccines as well as in structure-function studies.

Despite the availability of cholera vaccines in various forms since shortly after the first isolation of cholera vibrios by Robert Koch in 1883, a suitable cholera vaccine—one which is highly protective, economical, convenient, and without side effects—remains to be developed and deployed (reviewed in references 13, 14, and 27). The most promising current candidates include either living attenuated or killed vaccines administered perorally; both kinds have been evaluated in human clinical studies and found to be less than ideal in one or more aspects. A conjugate vaccine, consisting of detoxified vibrio lipopolysaccharide (LPS) covalently linked to cholera enterotoxin (CT), has also been prepared (22) but has not yet been evaluated in humans. A potential drawback is that the presence of free toxin remaining after the conjugation step necessitates additional purification and introduces some element of risk. Although CT is essential for the life-threatening diarrhea

of cholera, vaccines consisting of chemically inactivated CT administered parenterally, or of its immunodominant B-subunit protein administered perorally with killed vibrios, were minimally protective; in the latter instance, the results of an extensive field study suggested that the addition of the B subunit actually reduced the efficacy of the whole-cell vaccine (11). The B-subunit protein, by itself, has previously been shown to be a less effective immunogen than other forms of CT antigen, including the holotoxin itself (13, 14). However, the disease cholera is itself an effective immunizing process in which both antitoxic and antibacterial antibodies are produced locally and systemically (27), having been stimulated by the in vivo-grown vibrios and their products. It would thus appear that the most effective way to induce immunity would be to simulate the disease process without evoking its undesirable symptoms, i.e., by use of a live attenuated vaccine.

CT is a multimeric protein exotoxin. The A ("active") protomer and the pentameric B ("binding") subunits are encoded by a single transcriptional unit, *ctxAB* (36). When posttranslationally modified by proteolysis and reduction (21), the catalytic A₁ subunit of CT is capable of ADP-ribosylating

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the guanine nucleotide-binding protein, which regulates cell adenyl cyclase, leading to the severe diarrhea of cholera. The catalytic subunit of several ADP-ribosylating bacterial toxins share certain small regions of amino acid sequence identity (20, 41). Arg-9 is essential for enzymatic activity of pertussis toxin, and substitution of lysine for Arg-9 in the S-1 subunit of pertussis toxin abolishes ADP-ribosyltransferase activity and toxicity without altering a neutralizing epitope (8, 38). Similarly, the substitution of lysine for Arg-7 in the A subunit of CT, and in the A subunit of the CT-related heat-labile enterotoxin (LT) of *Escherichia coli*, eliminated ADP-ribosyltransferase activity (9, 29). Substitution of aspartic acid for Glu-110 or Glu-112 severely repressed ADP-ribosyltransferase activity of LT (29). Chemical mutagenesis that resulted in the substitution of lysine for Glu-112 caused a loss of toxicity and ADP-ribosyltransferase activity (46, 47). The protein did, however, interact with ADP-ribosylation factor (ARF), the 20-kDa guanine nucleotide-binding protein that serves as a GTP-dependent allosteric activator of CT and LT (34). Jobling and Holmes (24) have recently reported a variety of site-directed mutations of CT-A that had marked effects on holotoxin assembly and toxicity. To our knowledge, no one has yet substituted mutagenized *ctxA* for the wild-type gene in *Vibrio cholerae*.

Employing site-directed mutagenesis, in the present study we have created mutant CT-A subunit genes with codon substitutions for both Arg-7 and Glu-112, changes that eliminated ADP-ribosyltransferase activity. Genes encoding inactive analogs of CT (CT-1* and CT-2*, representing the two major epitopes of CT [13, 14, 31, 39]) were inserted into the chromosome of wild-type *V. cholerae* strains, including both serogroup O1 Inaba and Ogawa serotypes of El Tor and classical biotypes and the O139 (synonym Bengal) serogroup currently epidemic in India and Bangladesh (10, 40). The resulting recombinant strains may have potential as living attenuated vaccines, and the CT analogs could themselves be useful in conjugate vaccines and in structure-function studies.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids utilized are described in Table 1. The *E. coli* cells were grown at 37°C in Luria-Bertani broth medium (43). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added at the following concentrations (in micrograms per milliliter): ampicillin, 50; kanamycin, 50; chloramphenicol, 10; tetracycline, 12.5 (*E. coli*) or 5 (*V. cholerae*). *V. cholerae* was cultured in syncase broth (15) for electroporation or toxin production.

DNA preparation, manipulation, and analysis. Plasmid DNA was extracted from *E. coli* DH5 α cells by the alkaline lysis method of Birnboim and Doly (3). *V. cholerae* chromosomal DNA was prepared as described previously (2). Standard techniques (43) were used in the construction of recombinant plasmids. Restriction enzymes were generally obtained from Promega (Madison, Wis.), and digestions were done with buffers provided by the suppliers under the recommended conditions. Restriction fragments were electrophoresed in horizontal 0.8% agarose gels in TAE buffer (43) and stained with ethidium bromide (0.5 μ g/ml). Restriction fragments were isolated from low-melting-point agarose (FMC BioProducts, Rockland, Maine) gels, melted at 55°C, and used directly in ligation reactions. Site-directed mutagenesis was performed as described previously (9).

Southern blot transfers were performed in 20 \times SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) with MagnaGraph nylon

membranes (MSI, Westboro, Mass.). The probe, p3083 (5), was labelled with digoxigenin-11-dUTP by the random-primer method employed in the Genius System Kit (Boehringer Mannheim, Indianapolis, Ind.). Hybridizations were performed overnight at 55°C, and the membranes were washed at 55°C with a final stringency of 0.1 \times SSC plus 0.1% sodium dodecyl sulfate (SDS). Hybridizing bands were visualized by using the reagents and protocol for colorimetric detection provided in the Genius System Kit.

The *ctxAB* locus was amplified by PCR with the oligonucleotide primers 5'-dCTGTTAAACAAAGGGAGC-3' (*ctxA*) and 5'-dCGGTGCTTCTCATCATCG3' (*ctxB*), using approximately 0.5 μ g of chromosomal DNA and employing the reagents and protocol of the GeneAmp kit (Perkin Elmer Cetus, Norwalk, Conn.). Thirty cycles of amplification were carried out in a Perkin Elmer DNA Thermocycler with strand denaturation (1 min at 96°C), annealing (1.5 min at 49°C), and extension (1.5 min at 72°C). The amplified DNA products were cloned directly into the *HincII* site of pGEM3Zf(+).

Sequence analyses were carried out on an Applied Biosystems automated sequencer using dye terminators. The cloned PCR products were sequenced by dideoxy-chain termination reactions (44) with [α -³⁵S]dATP (NEN Research Products) and the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Synthetic oligonucleotide primers were synthesized by the University of Missouri DNA Core Facility or at the Amgen-Boulder facility.

Electroporation. The *V. cholerae* strains were transformed by electroporation, with slight modifications of a published procedure (32). Bacterial cells were grown to mid-log phase in 20 ml of syncase medium (15) at 37°C, harvested and washed three times in the electroporation buffer (272 mM sucrose, 7 mM Na₂HPO₄ [pH 7.4], 1 mM MgCl₂), and resuspended in 1 ml of the same buffer. After the plasmid DNA was added, the bacteria were subjected to electric shock (Bio-Rad Gene Pulser, 25- μ F capacitance, 2.5 kV) and allowed to recover in 1.5 ml of syncase medium at 37°C. The bacteria were pelleted, resuspended in 200 μ l of medium, and plated on meat extract agar plates containing the appropriate antibiotics.

Recombinant CT analog production. For CT* production, 500-ml flasks containing 100 ml of syncase medium supplemented with antibiotics were inoculated with 2×10^6 viable bacteria per ml and incubated overnight (20 h) at 30°C with shaking. The recombinant toxin analogs were isolated from culture supernatants and partially purified by Al(OH)₃ adsorption and elution (18).

ARF-stimulated NAD:agmatine ADP-ribosyltransferase activity. The NAD:agmatine ADP-ribosyltransferase assay was performed essentially as described previously (34). Four micrograms of partially purified recombinant toxins was incubated at 30°C for 10 min in 70 mM glycine-30 mM dithiothreitol, pH 8.0. Wild-type CT holotoxin was treated similarly. Assay mixtures contained 50 mM potassium phosphate, pH 7.5, 5 mM MgCl₂, 100 μ M GTP, 20 mM dithiothreitol, 0.1 mg of ovalbumin per ml, 100 μ M [adenine-U-¹⁴C]NAD (approximately 100,000 cpm), 3 mM dimyristoylphosphatidylcholine, 0.2% cholate, 10 mM agmatine, 0.5 μ g of recombinant ARF 6, and 0.5 μ g of wild-type or recombinant toxin in a 150- μ l total volume. After incubation for 1 h at 30°C, 50- μ l samples were transferred to columns of AG1-X2, which were washed five times with 1 ml of water each time. All eluates were collected for radioassay by liquid scintillation counting.

Toxicity. Toxicity was assayed in vitro using Y-1 adrenal tumor cells (42) essentially as described previously (31). Samples containing up to 100 ng of toxin antigen (as determined by a radial diffusion immunoassay [19]) were added in duplicate

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source (reference) ^a
<i>E. coli</i> DH5α	Host for recombinant plasmids	BRL
<i>V. cholerae</i>		
JBK70	<i>ctxAB</i> deletion derivative of El Tor, Inaba N16961	J. B. Kaper (28a)
CVD101	CT-A ⁻ CT-B ⁺ deletion derivative of classical, Ogawa 395	J. B. Kaper (28a)
CVD101/CT-1*	<i>ctxA</i> *B-1, derivative of CVD101, Kan ^r	This study
CVD101/CT-2*	<i>ctxA</i> *B-2, derivative of CVD101, Kan ^r	This study
CVD103	CT-A ⁻ CT-B ⁺ deletion derivative of classical, Inaba 569B	J. B. Kaper (28)
CVD103/CT-1*	<i>ctxA</i> *B-1, derivative of CVD103, Kan ^r	This study
CVD103/CT-2*	<i>ctxA</i> *B-2, derivative of CVD103, Kan ^r	This study
C6706	Wild-type El Tor, Inaba, from Peru, 1991	K. Wachsmuth, CDC
C6706/CT-1*	<i>ctxA</i> *B-1, <i>zot</i> mutant derivative of C6706, Kan ^r	This study
C6706/CT-2*	<i>ctxA</i> *B-2, <i>zot</i> mutant derivative of C6706, Kan ^r	This study
C7258	Wild-type El Tor, Ogawa, from Peru, 1991	K. Wachsmuth, CDC
C7258/CT-1*	<i>ctxA</i> *B-1, <i>zot</i> mutant derivative of C7258, Kan ^r	This study
C7258/CT-2*	<i>ctxA</i> *B-2, <i>zot</i> mutant derivative of C7258, Kan ^r	This study
SG25-1	Wild-type, O139 (synonym Bengal), from India, 1993	G. B. Nair (40)
SG25-1/CT-1*	<i>ctxA</i> *B-1, <i>zot</i> mutant derivative of SG25-1, Kan ^r	This study
SG25-1/CT-2*	<i>ctxA</i> *B-2, <i>zot</i> mutant derivative of SG25-1, Kan ^r	This study
Plasmids		
p3083	8.3-kb <i>Pst</i> I fragment containing <i>ctxAB</i> -2 from <i>V. cholerae</i> 3083 cloned into pBR328; Cml ^r Tet ^r	T. J. Brickman (5)
pJM17	5.1-kb <i>Eco</i> RI- <i>Pst</i> I fragment containing <i>ctxAB</i> -1 from <i>V. cholerae</i> 569B cloned into pBR322; Tet ^r	J. J. Mekalanos (36)
pCT-A*	1.0-kb <i>Eco</i> RI- <i>Hinc</i> II fragment containing <i>ctxA</i> * with Arg-7→Lys (CGG→AAG) and Glu-112→Gln (GAA→CAG) changes in pUC19; Amp ^r	This study
p3083*	Derivative of p3083 in which the 0.8-kb <i>Nde</i> I fragment has been replaced by the analogous <i>Nde</i> I fragment from pCT-A*; Cml ^r Tet ^r , CT-2*	This study
pCT-2*	Derivative of p3083* with a Kan ^r gene inserted into the <i>Nsi</i> I site within <i>zot</i> ; Cml ^r Tet ^r Kan ^r CT-2*	This study
p3083*-BstXI	Derivative of p3083* in which the 0.75-kb <i>Bst</i> XI fragment has been replaced by the analogous <i>Bst</i> XI fragment from pJM17; Cml ^r Tet ^r CT-1*	This study
pCT-1*	Derivative of p3083*- <i>Bst</i> XI with the Kan ^r gene inserted into the <i>Nsi</i> I site within <i>zot</i> ; Cml ^r Tet ^r Kan ^r CT-1*	This study
pGEM3Zf(+)	Amp ^r <i>lacZ</i> α	Promega
pUC19	Amp ^r <i>lacZ</i> α	BRL
p657	Amp ^r subclone of pBR322, a derivative of ColE1, with 1 kb of rickettsial DNA inserted into <i>Hind</i> III	G. A. McDonald, unpublished

^a BRL, Bethesda Research Laboratories; CDC, Centers for Disease Control and Prevention.

to microtiter wells containing cultured Y-1 cells. CT-1 and CT-2 (1 ng each) were used as positive controls, and the results were recorded after 6 h of incubation.

Enterotoxicity. The ability of recombinant toxin analogs to cause diarrhea, and the virulence of strains producing CT-1* and CT-2*, were assayed in infant rabbits by standard methods employed in our laboratory (16): toxin and toxin analogs were fed to rabbits in 5 ml of 1% Tris, pH 8.0, whereas the bacterial strains were inoculated intraintestinally. Choleraenic scores were calculated for experimental groups of three or more animals.

Antigens and antibodies. CT-1 and CT-2, used as standards, were purified in our laboratory from fermentor-grown cultures (18) of classical biotype, Inaba serotype *V. cholerae* strain 569B and El Tor biotype, Ogawa serotype strain 3083 T, respectively. Hyperimmune rabbit polyclonal antisera used in this study were previously raised in this laboratory against CT-1 A- and B-subunit proteins and against CT-2 B-subunit protein. Unlike the B-subunit proteins, the A subunits of the two

biotypes are identical (12). Monoclonal antibodies (MAbs) against B-subunit proteins used in this study were described previously and included anti-CT-1 (α-CT-1) and α-CT-2 classes I, III, and VI, α-H-LT class II, α-pDL-3 class IV, and α-S-LT class V (26). MAbs KB9 and 8E11 against the A subunit of CT-1 were also used (17).

Microzone electrophoresis. The recombinant proteins were analyzed by using the Microzone cellulose acetate electrophoresis system (Beckman Instruments Inc., Fullerton, Calif.) (6).

SDS-PAGE and Western blotting (immunoblotting). Samples of proteins were subjected to polyacrylamide gel electrophoresis (PAGE) in 4% stacking and 15% separating polyacrylamide gels and transferred onto nitrocellulose membranes as described previously (7).

G_{M1} binding. G_{M1} binding was evaluated by the Ouchterlony double diffusion in gel method. The checkerboard immunoblotting procedure (25), the G_{M1} modification (26), and the G_{M1} enzyme-linked immunosorbent assay (G_{M1}-ELISA) have been described (17).

RESULTS

Construction of plasmids encoding CT-2* and CT-1*. Plasmid pCT-A* was constructed by subcloning a synthetic oligonucleotide linker with an Arg-7→Lys codon substitution (CGG→AAG) into the mutated *ctxA* gene containing a Glu-112→Gln (GAA→CAG) mutation, thus combining both codon substitutions. An 8.3-kb *Pst*I fragment carrying the *ctxAB* genes from *V. cholerae* 3083, a CT-2 producer, was previously cloned into pBR328 (p3083) (5). To place the mutated CT-A subunit gene in a functional genetic environment for toxin production, the wild-type *ctxA* gene of p3083 was replaced with the mutated *ctxA** gene by exchange of the analogous 0.8-kb *Nde*I restriction fragment containing most of the CT-A subunit gene, thus generating p3083*. The CT gene from *V. cholerae* 569B, which produces CT-1, was previously cloned into pBR322 (pJM17) (36). To combine the mutated *ctxA** gene with the gene encoding the B subunit of CT-1, the 0.75-kb *Bst*XI fragment of pJM17, containing the majority of *ctxB-1*, was used to replace the *Bst*XI fragment of p3083*. The presence of both codon mutations in plasmids p3083* and p3083*-*Bst*XI, now encoding the toxin analogs CT-2* and CT-1*, respectively, was confirmed by DNA sequencing. Further, a Kan^r gene cassette was inserted into the *Nsi*I site in the *zot* gene (1), which is located immediately upstream of *ctxA**B, in p3083* and p3083*-*Bst*XI, thus generating pCT-2* and pCT-1*, respectively.

Construction of *V. cholerae* strains carrying the inactive toxin gene. Plasmids pCT-1* and pCT-2* were introduced into wild-type El Tor biotype *V. cholerae* C6706 and C7258, O139 strain SG25-1, and mutant classical vibrio strains CVD101 and CVD103 by electroporation. Strains carrying the plasmids were then transformed by a second, incompatible plasmid, p657, and selected for Amp resistance. Kan^r vibrios that had lost all plasmid resistance markers (and thus should carry the Kan^r cassette in the chromosomal *zot* gene after a homologous recombination event) were isolated that showed no toxin activity in the Y1 assay. Southern blot analysis of restriction enzyme-digested chromosomal DNA of the recombinant strains and the wild-type parents demonstrated the expected larger size of the restriction fragments carrying the CT gene region in the mutant strains (Fig. 1). Interestingly, SG25-1 wild-type DNA differs from that of the Peru isolates in both the *Pst*I and *Hind*III digests, consistent with another report suggesting an amplification of the CT gene cassette in the O139 strains (48). However, the mutant strains created here show band patterns similar to those of the mutant strains created from the Peru isolates, indicating the deletion of all additional copies of CT genes. Since the Kan^r gene cassette introduces a *Hind*III site in the mutant DNA that is not present in the parental DNA, an additional band is observed in those lanes (Fig. 1). To confirm the presence of both point mutations in the *ctxA* genes and the successful recombination of the *ctxB* genes encoding the B subunit of CT-1 in C6706/CT-1*, C7258/CT-1*, and SG25-1/CT-1*, the entire *ctxAB* operon was amplified by PCR from both the mutant and parental wild-type strains; DNA fragments of approximately 1.2 kb were amplified, cloned, and partially sequenced. In all mutant strains, codon substitutions for both Arg-7 and Glu-112 were present in the *ctxA** genes. As expected, differences in nucleotide sequences between the *ctxB* genes encoding the B subunits of CT-1 and CT-2 were found: the *ctxB* genes from C6706/CT-1*, C7258/CT-1*, and SG25-1/CT-1* encode His-18 and Thr-47 (CT-1), whereas those of C6706/CT-2*, C7258/CT-2*, and SG25-1/CT-2* encode Tyr-18 and Ile-47 (CT-2) (12, 39).

Toxin analog characterization. Strain JBK70 was trans-

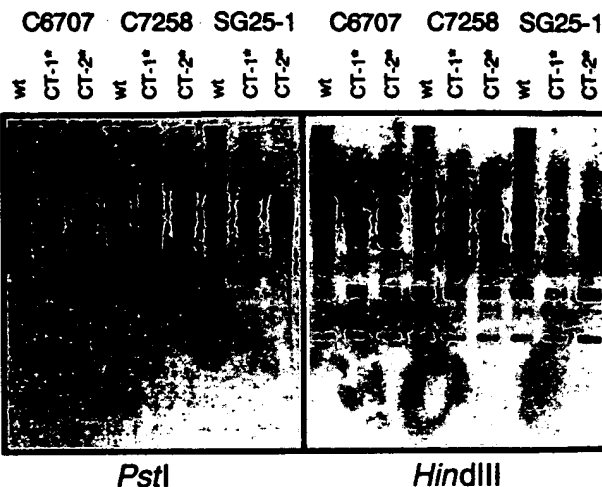


FIG. 1. Southern blot analysis, using p3083 as a probe, of chromosomal DNA from *V. cholerae* wild-type (wt) strains and their derivatives encoding recombinant toxins (CT-1* or CT-2*) digested with restriction enzymes *Pst*I and *Hind*III. The *Pst*I digest reveals that the lower (smaller) band increases by approximately 1.3 kb due to the insertion of a Kan^r cassette in *zot*. SG25-1, an O139 serotype strain, differs from the El Tor biotype O1 strains from Peru, C6707 and C7258, but assumes the same pattern after recombination. The insertion of the Kan^r cassette introduces an additional *Hind*III site. SG25-1 again differs from the El Tor biotype strains and assumes the same pattern after recombination.

formed with plasmids pCT-1* and pCT-2* for the purpose of producing toxin analogs for analysis. The yields of analog proteins from plasmid-carrying strain JBK70 ranged from approximately 0.15 to 0.25 µg/ml. Examination by Western blotting of the toxin analogs, CT-1* and CT-2*, partially purified with Al(OH)₃, revealed unnicked A* (28 kDa), A₁* (21 kDa), and B (11.5 kDa) protein bands (Fig. 2). Because CT-1*, CT-2, and CT-2* were rapidly isolated, the A subunit

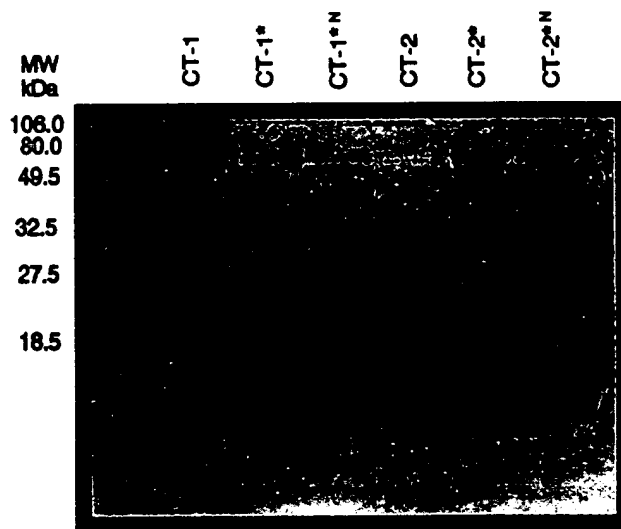


FIG. 2. Western blot analysis of partially purified CT analogs. The A-subunit proteins of CT-1*, CT-2, and CT-2*, prepared rapidly, were largely unnicked. CT-1*N and CT-2*N were pretreated with trypsin to form CT-A*. The blots were developed using specific polyclonal rabbit α-A and α-B (1:1,000) prepared in this laboratory.

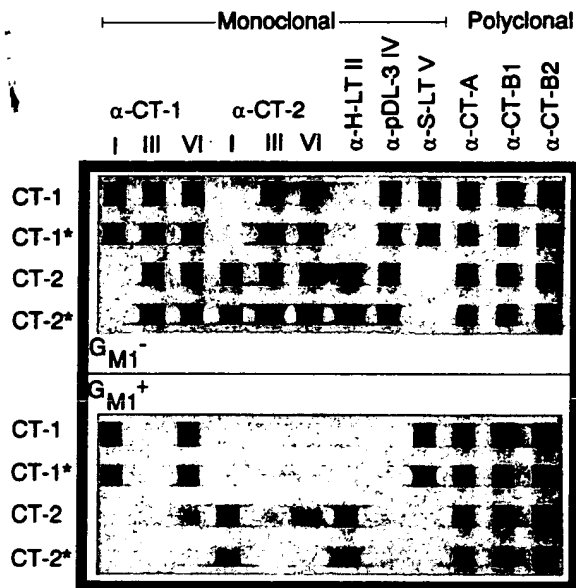


FIG. 3. Checkerboard immunoblotting analysis of the reactivity of CT and CT* with monoclonal and polyclonal antibodies and the effect of G_{M1}. α-CT-1 class I recognizes only CT-1 and CT-1*. α-CT-1 class III recognizes both CT-1 and CT-2 and their derivatives but is blocked by G_{M1}. α-CT-1 class VI recognizes both epitopes, but its reactivity with CT-2 is blocked by G_{M1}. α-CT-2 class I recognizes only CT-2 and its derivative. α-CT-2 class III and class VI recognize both epitopes and are blocked by G_{M1}. α-H-LT class II recognizes only CT-2 and CT-2*. α-pDL-3 class IV recognizes both epitopes and is blocked by G_{M1}. α-S-LT class V recognizes only CT-1 and CT-1*. Polyclonal antibodies react with both epitopes and their derivatives similarly.

was predominantly unnicked (4). When the toxin analogs were treated overnight at 24°C with 1.8 μg of trypsin per ml (21), they were fully converted to a nicked form (Fig. 2).

The partially purified toxin analogs migrated identically to wild-type CT in cellulose acetate membrane electrophoresis, and the single protein bands were stained with both α-A and α-B polyclonal antibodies. To determine whether the amino acid substitutions affected the immunological properties of these toxin analogs, antitoxin MAbs (26) were used to compare the reactivity of the CT* proteins with wild-type toxins. The reactions of the toxins and their analogs with various antibodies in checkerboard immunoblotting are shown in Fig. 3. In contrast to CT-2 and CT-2*, CT-1 and CT-1* are recognized by α-CT-1 class I and α-S-LT class V MAbs but are not recognized by α-CT-2 class I, and α-H-LT class II. The reactions of α-CT-1 class III, α-CT-2 class III and VI, and α-pDL-3 class IV MAbs with both CT epitopes and their derivatives were blocked by G_{M1} (Fig. 3). However, the reaction of α-CT-1 class VI MAb was blocked by G_{M1} only with CT-2 and CT-2*. Polyclonal antibodies recognized mutant and wild-type proteins equally well. Thus, the immunological properties of the CT analogs, by these criteria, were identical to those of wild-type toxins.

The ability of the B subunits of the toxin analogs to recognize and react with G_{M1} ganglioside was further examined by G_{M1} immunodiffusion-in-gel analysis (G_{M1}-Ouchterlony). Recombinant toxin analogs formed precipitation bands with G_{M1} ganglioside that were indistinguishable from those of wild-type CTs. Results of G_{M1}-ELISA indicated that the toxin analogs formed intact heteropolymers. The toxin analogs reacted well with polyclonal α-A and polyclonal α-B sera in

this assay, showing that, in binding to G_{M1}, the B pentamer carried the A subunit protein with it. However, the reactions of α-A subunit MAbs with both CT-1* and CT-2* were weaker than those with CT-1 and CT-2 standards: i.e., the optical densities for the reactions between hybridoma supernatant (1:10) with approximately 100 ng of analog antigen ranged from 1.4- to 4-fold lower than those with the standards (e.g., 0.16 to 0.3 versus 0.43 to 0.65).

The purified toxin analogs, at up to 100-fold higher concentrations, failed to elicit the typical rounding of cultured Y-1 adrenal tumor cells that was observed with wild-type toxins. Additionally, the toxin analogs possessed no NAD:agmatine ADP-ribosyltransferase activity in the presence of ARF. Infant rabbits infected with 10⁶ to 10⁷ wild-type live vibrios or fed 5 μg of CT showed typical ante mortem symptoms or died of diarrhea. Significantly, none of the rabbits infected with recombinant *V. cholerae* strains producing CT* or fed 25 μg of CT* had diarrhea or fluid accumulation in the small intestines (choleraemic score, <1). *V. cholerae* organisms were recovered in practically pure culture from the intestines of the rabbits challenged with wild-type and recombinant strains.

DISCUSSION

The ultimate goal of this work is the development of a cholera vaccine which is effective (preferably in a single dose), economical, convenient, and suitably nonreactogenic. For reasons which have been discussed elsewhere (13, 14, 27), none of the many candidate vaccines which have been developed in the past has met all of these criteria; they have been insufficiently potent, too reactogenic, too expensive and inconvenient, or exhibited a combination of these deficiencies. One promising vaccine candidate, CVD103-HgR (a CT-A⁻ CT-B⁺ mutant of classical Inaba strain 569B), is well tolerated but, like its parent strain, poorly colonizes the gut; high doses are required (33), and it has a diminished protective efficacy against the more prevalent El Tor biotype (28). This reduced effectiveness against the El Tor biotype is yet unexplained but could relate to the fact that it is an A⁻ B⁺ strain (i.e., it has no CT-A antigen); further, the B-subunit protein it produces is of the CT-1 epitope, whereas most El Tor vibrios (except the Gulf Coast clone [personal observations]) produce CT-2. In addition, CVD103-HgR does not produce the cell-associated mannose-sensitive hemagglutinin that is characteristic of El Tor biotype strains (23) and, because of somatic antigen differences, it is likely to be ineffective against the new O139 serogroup strains.

It is not clear why CT antigens containing A-subunit protein (e.g., the holotoxin or procholeraenoid) elicit stronger immune responses than the B-subunit pentamer (choleraenoid) (37). The fact that procholeraenoid (a heat-induced aggregate of CT) was minimally toxic, but equally as immunogenic as the holotoxin, would suggest that ADP-ribosyltransferase activity was not responsible for its enhanced immunogenicity. However, these observations are complicated by its high molecular weight and slight residual CT-like activity. CT has been recognized since the pioneer study of Northrup and Fauci (35) to be a powerful immunomodulator, having both adjuvant and immunosuppressive effects that are dependent on dose and time of administration. Lycke et al. (30) have recently reported that analog LT (with lysine substituted for Glu-112), lacking ADP-ribosyltransferase activity, like CT-B but unlike CT or LT, had no adjuvant activity for keyhole limpet hemocyanin. These investigators concluded that adjuvanticity was directly linked to ADP-ribosyltransferase activity, but since the amino acid substitution is rather nonconservative, altering the

pl of the protein (47), other explanations are clearly feasible. The immunogenicity of the analog and native proteins were not compared in the study by Lycke et al. (30).

In the present investigation, conservative substitutions for both Arg-7 and Glu-112, each of which have been shown to eliminate ADP-ribosyltransferase activity, were introduced as codon substitutions into the gene encoding the catalytic A subunit of CT. The mutated *ctxA** gene was joined with cloned *ctxB* genes from classical and El Tor biotype *V. cholerae* strains to produce the recombinant toxin analogs CT-1* and CT-2*, respectively. The toxin analogs were partially purified and found to lack (i) toxicity for cultured adrenal tumor cells, (ii) ADP-ribosyltransferase activity in vitro, and (iii) diarrheagenic activity (in infant rabbits). They retained their ability to form heteropolymers and to interact with G_{M1}, and they reacted similarly to the wild type with MAbs against CT-B. However, their reactivity with α -CT-A MAbs was weaker than that of the wild type, perhaps reflecting some conformational difference in an epitope or epitopes, recognized by the α -CT-A MAbs, which are dependent on Arg-7 and/or Glu-112.

The genes encoding the recombinant toxin analogs were introduced by homologous recombination into the chromosomes of El Tor biotype *V. cholerae* C6706 (Inaba) and C7258 (Ogawa), recently isolated in Peru; SG25-1, an O139 serotype strain from the current epidemic in India; and classical biotype mutant vibrios CVD101 and CVD103, CT-A⁻ CT-B⁺ derivatives of strains 396 (Ogawa) and 569B (Inaba), respectively. Southern blot analyses indicated the insertion of the kanamycin gene in *zot*. Amplification of the CT genes by PCR and partial nucleotide sequencing confirmed the presence of both codon substitutions in the *ctxA** genes and the appropriate *ctxB* genes. Our observations do not as yet permit the likely conclusion that the original *ctxB-1* is retained in the classical biotype CT-2*-producing mutants; i.e., they could conceivably be producing chimeras of CT*-B-1 and CT*-B-2 which could be advantageous. Studies are in progress to enable us to make this determination.

The El Tor and O139 recombinant strains were avirulent in the infant rabbit animal model. Whether they are suitably avirulent for use in humans can be determined only by trial in volunteers. Previous studies with small numbers of volunteers have demonstrated that Tox⁻ mutants (e.g., JBK70), although diarrheagenic by themselves, elicited significant protection against subsequent challenge with the virulent wild type (28a). The mechanisms of this protection are not clear, although an antibacterial (anti-LPS) component was presumed to be involved since the challenged volunteers yielded fewer vibrios on coproculture. Whether engineered strains expressing toxin analogs containing both A and B subunits would provide better and more durable homologous immunity remains to be tested. They could potentially also offer better cross-protection against related enterotoxins if A*B is more effective than B alone as an antigen and adjuvant. It is also evident from the recent volunteer studies with Tox⁻ mutants that, even though CT is the factor responsible for the severe, life-threatening diarrhea of cholera, the vibrios have other mechanisms for causing milder forms of diarrhea. CVD110, a mutant from El Tor biotype Ogawa serotype strain E7946 deleted for all known virulence factors, was still capable of causing diarrhea (mean stool volume of 861 ml) in 7 of 10 volunteers (45). The act of colonization may itself be diarrheagenic; alternatively, another, yet undiscovered, cholera enterotoxin may be responsible. As the capability of mutant *V. cholerae* strains to cause diarrhea is still an unpredictable event, volunteer studies with the mutants described herein may provide useful information. For example, derivatives of serogroup O139 have yet to be

tested in humans, and it is conceivable that the recombinant Peru El Tor biotype strains may be more suitable than strains previously tested. Additional mutations may be still needed, and it may be desirable to mix some of the recombinant strains. The analog toxins will be useful for future studies to evaluate the role of ADP-ribosyltransferase activity in the immunogenicity and adjuvanticity of CT. Nontoxic analogs consisting of an altered A subunit and CT-B-1 and CT-B-2 may be useful as oral or conjugate parenteral vaccines (22).

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REFERENCES

1. Baudry, B., A. Fasano, J. Ketley, and J. B. Kaper. 1992. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. *Infect. Immun.* 60:428-434.
2. Berman, M. L., L. W. Enquist, and T. J. Silhavy. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
4. Booth, B. A., M. Boesman-Finkelstein, and R. A. Finkelstein. 1984. *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. *Infect. Immun.* 45:558-560.
5. Brickman, T. J., M. Boesman-Finkelstein, R. A. Finkelstein, and M. A. McIntosh. 1990. Molecular cloning and nucleotide sequence analysis of cholera toxin genes of the CtxA⁻ *Vibrio cholerae* strain Texas Star-SR. *Infect. Immun.* 58:4142-4144.
6. Briere, R. O., and J. D. Mull. 1964. Electrophoresis of serum proteins with cellulose acetate. *Am. J. Clin. Pathol.* 42:547-551.
7. Burnette, W. N. 1981. "Western blotting", electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
8. Burnette, W. N., W. Cieplak, V. L. Mar, K. T. Kaljot, H. Sato, and N. M. Keith. 1988. Pertussis toxin S1 mutant with reduced enzyme activity and a conserved protective epitope. *Science* 242:72-74.
9. Burnette, W. N., V. L. Mar, B. W. Platler, J. D. Schlotterbeck, M. D. McGinley, K. S. Stoney, M. F. Rohde, and H. R. Kaslow. 1991. Site-specific mutagenesis of the catalytic subunit of cholera toxin: substituting lysine for arginine 7 causes loss of activity. *Infect. Immun.* 59:4266-4270.
10. Cholera Working Group. 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. *Lancet* 342:387-390.
11. Clemens, J. D., D. A. Sack, J. R. Harris, F. van Loon, J. Chakraborty, F. Ahmed, M. R. Rao, M. R. Khan, M. Yunus, N. Huda, B. F. Stanton, B. A. Kay, S. Walter, R. Eeckels, A.-M. Svennerholm, and J. Holmgren. 1990. Field trial of cholera vaccines in Bangladesh: results from three-year follow-up. *Lancet* 335:270-273.
12. Dams, E., M. de Wolf, and W. Dierick. 1991. Nucleotide sequence analysis of the CT operon of the *Vibrio cholerae* classical strain 569B. *Biochim. Biophys. Acta* 1090:139-141.
13. Finkelstein, R. A. 1992. Cholera enterotoxin (choleragen): a historical perspective, p. 155-187. *In* D. Barua and W. B. Greenough III (ed.), *Current topics in infectious diseases: Cholera*. Plenum Medical Publishers, New York.
14. Finkelstein, R. A. 1993. Why do we not yet have a safe, effective and economical vaccine against cholera?, p. 137-153. *In* A. F. Pestana de Castro and W. F. Almeida (ed.), *Cholera on the American continents*. International Life Sciences Institute/ILSA Press, Washington, D.C.
15. Finkelstein, R. A., P. Athasampunna, M. Chulasamaya, and P.

- Charunmethee. 1966. Pathogenesis of experimental cholera: biological activities of purified procholera A. J. Immunol. 96:440-449.
16. Finkelstein, R. A., M. Boesman-Finkelstein, Y. Chang, and C. C. Häse. 1992. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. Infect. Immun. 60:472-478.
 17. Finkelstein, R. A., M. F. Burks, A. Zupan, W. S. Dallas, C. O. Jacob, and D. S. Ludwig. 1987. Epitopes of the cholera family of enterotoxins. Rev. Infect. Dis. 9:544-561.
 18. Finkelstein, R. A., K. Fujita, and J. J. LoSpalluto. 1971. Procholeraenoid: an aggregated intermediate in the formation of choleraenoid. J. Immunol. 107:1043-1051.
 19. Finkelstein, R. A., and J. J. LoSpalluto. 1969. Pathogenesis of experimental cholera: preparation and isolation of choleraen and choleraenoid. J. Exp. Med. 130:185-202.
 20. Gill, D. M. 1988. Sequence homologies among the enzymatically active portions of ADP-ribosylating toxins. Zentralbl. Bakteriol. Suppl. 17:315-323.
 21. Gill, D. M., and R. S. Rappaport. 1979. Origin of the enzymatically active A1 fragment of cholera toxin. J. Infect. Dis. 139:674-680.
 22. Gupta, R. K., S. C. Szu, R. A. Finkelstein, and J. B. Robbins. 1992. Synthesis, characterization and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. Infect. Immun. 60:3201-3208.
 23. Hanne, L. F., and R. A. Finkelstein. 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. 36:209-214.
 24. Jobling, M. G., and R. K. Holmes. 1993. Molecular genetic studies of the structure and function of the A subunit of cholera enterotoxin, p. 83-87. In Proceedings of the 29th Joint Conference, U.S.-Japan Cooperative Medical Science Program, Cholera and Related Diarrheal Diseases. National Institutes of Health, Bethesda, Md.
 25. Kazemi, M., and R. A. Finkelstein. 1990. Checkerboard immunoblotting (CBIB): an efficient, rapid, and sensitive method of assaying multiple antigen/antibody cross-reactivities. J. Immunol. Methods 128:143-146.
 26. Kazemi, M., and R. A. Finkelstein. 1990. Study of epitopes of cholera enterotoxin-related enterotoxins by checkerboard immunoblotting. Infect. Immun. 58:2352-2360.
 27. Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements. 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. 47:510-550.
 28. Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. A. Losonsky, C. O. Tacket, B. D. Tall, and S. J. Cryz. 1988. Safety, immunogenicity and efficacy of recombinant live oral cholera vaccines, CVD103 and CVD103-HgR. Lancet II:467-470.
 - 28a. Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, J. G. Morris, M. L. Clements, R. E. Black, B. Tall, and R. Hall. 1988. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect. Immun. 56:161-167.
 29. Lobet, Y., C. W. Cluff, and W. Cieplak, Jr. 1991. Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the A subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. 59:2870-2879.
 30. Lycke, N., T. Tsuji, and J. Holmgren. 1992. The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur. J. Immunol. 22:2277-2281.
 31. Marchlewicz, B. A., and R. A. Finkelstein. 1983. Immunologic differences among the cholera/coli family of enterotoxins. Diagn. Microbiol. Infect. Dis. 1:129-138.
 32. Marcus, H., J. M. Ketley, J. B. Kaper, and R. K. Holmes. 1990. Effects of DNase production, plasmid size, and restriction barriers on transformation of *Vibrio cholerae* by electroporation and osmotic shock. FEMS Microbiol. Lett. 68:149-154.
 33. Migasena, S., P. Pitisuttitham, B. Prayurahong, P. Suntharasa-mai, W. Supanaranond, V. Desakorn, U. Vongsthongsri, B. Tall, J. Ketley, G. Losonsky, S. Cryz, J. B. Kaper, and M. M. Levine. 1989. Preliminary assessment of the safety and immunogenicity of live oral cholera vaccine strain CVD103-HgR in healthy Thai adults. Infect. Immun. 57:3261-3264.
 34. Moss, J., S. J. Stanley, M. Vaughan, and T. Tsuji. 1993. Interaction of ADP-ribosylation factor with *Escherichia coli* enterotoxin that contains an inactivating lysine 112 substitution. J. Biol. Chem. 268:6383-6387.
 35. Northrup, R. S., and A. S. Fauci. 1972. Adjuvant effect of cholera enterotoxin on the immune response of the mouse to sheep red blood cells. J. Infect. Dis. 125:672-673.
 36. Pearson, G. D. N., and J. J. Mekalanos. 1982. Molecular cloning of *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA 79:2976-2980.
 37. Pierce, N. F., W. C. Cray, Jr., J. B. Sacci, Jr., J. P. Craig, R. Germanier, and E. Führer. 1983. Procholeraenoid: a safe and effective antigen for oral immunization against experimental cholera. Infect. Immun. 40:1112-1118.
 38. Pizza, M., A. Covacci, A. Bartoloni, M. Perugini, L. Nencioni, M. T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, F. Giovannoni, R. Olivieri, J. T. Barbieri, H. Sato, and R. Rappuoli. 1989. Mutants of pertussis toxin suitable for vaccine development. Science 246:497-500.
 39. Qu, Z.-H., and R. A. Finkelstein. 1993. Checkerboard immunoblotting recognizes twenty epitopes among the B subunit proteins of the cholera enterotoxin family. Electrophoresis 14:899-901.
 40. Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazono, A. Pal, and Y. Takeda. 1993. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet 341:703-704.
 41. Rappuoli, R., and M. Pizza. 1991. Structure and evolutionary aspects of ADP-ribosylating toxins, p. 1-21. In J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, Inc., San Diego, Calif.
 42. Sack, D. A., and R. B. Sack. 1974. Test for enterotoxigenic *Escherichia coli* using Y-1 adrenal cells in miniculture. Infect. Immun. 11:334-336.
 43. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 44. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
 45. Tacket, C. O., G. Losonsky, J. P. Nataro, S. J. Cryz, R. Edelman, A. Fasano, J. Michalski, J. B. Kaper, and M. M. Levine. 1993. Safety and immunogenicity of live oral cholera vaccine candidate CVD110, a $\Delta ctxA \Delta zot \Delta ace$ derivative of El Tor Ogawa *Vibrio cholerae*. J. Infect. Dis. 168:1536-1540.
 46. Tsuji, T., T. Inoue, A. Miyama, and N. Noda. 1991. Glutamic acid-112 of the A subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* is important for ADP-ribosyltransferase activity. FEBS Lett. 291:319-321.
 47. Tsuji, T., T. Inoue, A. Miyama, K. Okamoto, T. Honda, and T. Miwatani. 1990. A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in a loss of its toxic activity. J. Biol. Chem. 265:22520-22525.
 48. Waldor, M. K., and J. J. Mekalanos. 1993. Molecular analysis of virulence determinants carried by the "Bengal" strain of *Vibrio cholerae* O139 and construction of vaccine prototypes, p. 42-46. In Proceedings of the 29th Joint Conference, U.S.-Japan Cooperative Medical Science Program, Cholera and Related Diarrheal Diseases. National Institutes of Health, Bethesda, Md.

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From Cholera Toxin to Subunit Vaccines

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Introduction

With his description of the enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae* in 1959, S. N. De set in motion a dynamic new era of cholera research. Through the molecular characterization of cholera toxin and its mode of action the pathogenesis of cholera became exceptionally well understood. This in its turn has led to the development of oral vaccines against both cholera and diarrhea caused by enterotoxinogenic *Escherichia coli*.

The possibility that cholera was a toxin-mediated disease had been postulated already in 1884 by Robert Koch. However, this concept soon fell in general disregard, when both Koch himself and others were unable to elicit cholera-like disease by injecting (*parenterally*) culture medium from *Vibrio comma* (*cholerae*) and it was assumed that toxins had to act via the bloodstream. The pathogenesis of cholera remained quite obscure for the next half-century, until in 1959, the situation changed dramatically. From India S. N. De could then report that a cell-free culture filtrate of *V. cholerae* after addition to the *mucosal* side of a ligated loop of the small intestine in a rabbit gave rise to fluid accumulation in the exposed loop¹.

This discovery started a new era in cholera research focussing on the characterization of the putative "cholera toxin" that was implied by De's finding. Within a short time, Panse and Dutta² described the excretion of toxin in the stool of cholera patients, and Craig³ both confirmed this and could demonstrate that cholera patients when recovering from disease had started to produce cholera toxin-neutralizing antibodies. These observations set the scene for the subsequent "modern history" of cholera toxin research, which in the ten-year period 1969–1978 made the pathogenesis of cholera exceptionally well understood. Furthermore, during the same period, certain strains of *Escherichia coli* and

various other bacterial species were found to cause diarrhoeal disease by producing analogous enterotoxins. This made cholera the prototype of a much larger group of "enterotoxic enteropathies" that were found to produce several hundred millions of diarrheal disease episodes each year. Based on the new knowledge about the enterotoxins and their mode of action, novel principles were also outlined which gave promise of the possibility to develop more effective diagnostic, therapeutic as well as preventive methods for cholera and the related enterotoxic enteropathies.

In 1978 a Nobel symposium was held in Stockholm to review and discuss the remarkably rapid progress in knowledge about "Cholera and Related Diarrhoeas" that had taken place in the past two decades⁴. S. N. De participated in this conference and could then take pleasure in the fact that his early discovery of the rabbit loop-active toxic factor had not only stimulated a new and successful line of research on cholera and other diarrhoeal disorders with great promise for future practical medical applications, but it had as well provided a widely used biological research tool to biomedical scientists in many different disciplines⁴.

Structure and Action of Cholera Toxin

As mentioned, many of the discoveries that defined the role of cholera toxin in the molecular pathogenesis of cholera were made in the ten-year period 1969–1978. Among the most important "chapters" were the ones listed below in a more or less chronological order:

- (i) The identification in 1969–71 by Field, Greenough, Sharp and others, of the strong, practically irreversible stimulatory effect of cholera toxin on the adenylate cyclase/cyclic AMP system and of the link between cyclic AMP and intestinal electrolyte and fluid secretion;

(ii) The purification of cholera toxin by Finkelstein and LoSpalluto and others in 1969–70, and the subsequent elucidation by Holmgren, Cuatrecasas, S. van Heyningen and others in 1972–74 of the A-B subunit structure of cholera toxin and the different role of the two types of subunit in the toxin function: the “light” B subunits were shown to provide tight, high-affinity binding of the toxin to cells and the “heavy” A subunit to mediate the direct cytotoxic action on intestinal adenylate cyclase leading to cyclic AMP formation and electrolyte and fluid secretion;

(iii) The identification in 1973–75 by W. van Heyningen, Holmgren and Svennerholm, Cuatrecasas and others of the GM1 ganglioside as the cell membrane receptor for cholera toxin;

(iv) The elucidation by Gill, Moss and Vaughan, Selinger and others in 1977–78 of the intracellular enzymic mechanism by which the cholera toxin A subunit activates the adenylate cyclase system, involving NAD hydrolysis with ADP-ribosylation of the N_s (G_s) regulatory subunit of adenylate cyclase.

I will not review here in any further detail these and the many additional contributions that led to

cholera toxin soon becoming perhaps best defined of all bacterial toxins; several comprehensive reviews with appropriate references exist on this subject (see e.g. 4, 5). For the main topic of this article, which is to describe how the new knowledge about cholera toxin has led to the recent development of oral subunit vaccines against cholera and related diarrhoeal disorders, it is sufficient to recite that by the end of the 1970s a fairly detailed picture of the pathogenesis of cholera had emerged based on the new understanding of cholera toxin (see Figure 1), which came also to guide the new era of vaccine development.

New Cholera Vaccines

There is a great need for an effective cholera vaccine. Cholera remains an important cause of illness and death in many parts of the world, especially in Asia but also in many parts of Africa and the Middle East. Although cholera can be treated simply by oral and intravenous rehydration, diarrhoea treatment centers are still scarce in areas endemic for cholera, and use of an effective vaccine may be the most promising possibility to control the disease. However, the parenteral whole-cell cholera vaccines that have existed since almost a century to prevent cholera are no longer regarded useful from a public health standpoint, mainly because of the short duration of protection they provide. Several field trials since 1960 have shown that these vaccines usually protect older people for only a few months and fail to protect many young children at all⁴.

The identification of the critical role of a toxin in the pathogenesis of cholera led to great expectations that a more effective cholera vaccine could be developed based on use of a toxoid. However, a large field trial with a parenterally administered glutaraldehyde toxoid vaccine in Bangladesh in the mid-1970s failed to give any better protection than that achieved by previous parenteral vaccines. This did not come as a total surprise, however, since during the time it took to organize, execute and evaluate the trial, it had become more clearly apparent from animal studies that protective cholera immunity did not depend on serum antibodies that were mainly stimulated by the parenteral vaccination, but rather on mucosal secretory IgA antibodies produced locally in the gut, which are only inefficiently stimulated by parenteral antigen administration⁶. Therefore, since the late 1970s, attention has turned instead to development of oral vaccines that could stimulate intestinal immunity more efficiently.

The different “modern” approaches towards

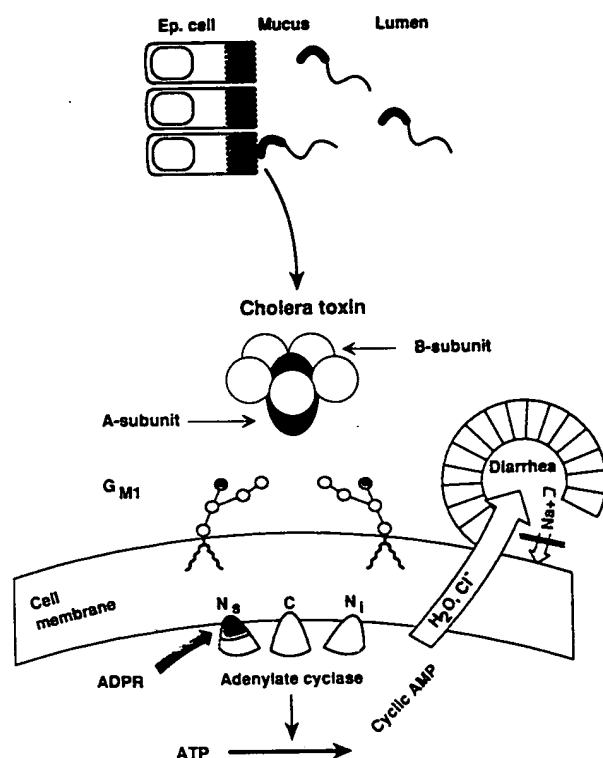


Figure 1. Pathogenesis of cholera and the action of cholera toxin (for explanations, see text).

development of cholera vaccines all have taken their departure from the new insights into the mechanisms of disease and immunity in cholera achieved during the 1970s and early 1980s.

Firstly, the clarification of the subunit structure of cholera toxin and of the role in different subunits in the toxin action immediately suggested a way to prepare a safe and highly immunogenic "toxoid" consisting of the purified cholera B subunits. Studies showed that the purified B subunit portion of cholera toxin was entirely devoid of toxicity, yet it contained selectively the protective toxin epitopes against which neutralizing antitoxin antibodies were directed. Indeed, it was found that the B subunit is particularly well suited to oral immunization because it retains the ability to bind to the intestinal epithelium, which has been shown to be important for stimulating mucosal immunity in animals, including local immunological memory⁶.

Another important line of vaccine research has focused on the gut mucosal immune system and how it can be stimulated by immunogens. These studies have defined the importance of locally produced IgA antibodies and IgA immunologic memory for protection against cholera, and they have shown that the oral route of vaccine administration is usually superior to the parenteral route in both priming and boosting the mucosal immune system⁶.

A third important observation guiding the design of the new cholera vaccines has concerned the synergistic cooperation between antitoxic and antibacterial immune mechanisms in cholera. Two main protective antibodies have been identified, one being directed against the *V. cholerae* cell wall lipopolysaccharide (LPS) and the other against the cholera toxin B subunits. Either of these two types of antibody can confer protection against disease by inhibiting bacterial colonization and toxin binding, respectively; when present together in the gut they can have a strongly synergistic protective effect⁶.

The intense vaccine development and research efforts during the last 10-year period have now provided several oral vaccine candidates based on either non-living bacteria and purified B subunit antigen or on live attenuated mutants of *Vibrio cholerae* O1 producing the B subunit.

The Oral B Subunit-Whole Cell Vaccine

The composition of this vaccine was designed to safely provide the key antigens for evoking protective antitoxic and antibacterial mucosal immunity against *V. cholerae* O1 of different serotypes (Inaba and Ogawa) and biotypes (classical and *El Tor*). As

mentioned, the B subunit component is completely non-toxic and is an exceptionally potent oral immunogen because of its ability to bind to the intestinal mucosa. The whole-cell (WC) component provides protective LPS antigens as well as heat-labile bacterial antigens that may add further to the antibacterial immunogenic effect.

The B-WC vaccine is given orally together with an alkaline buffer (provided by an effervescent tablet) to protect the vaccine during passage through the stomach. The vaccine has been extensively tested in several clinical trials in Swedish, Bangladeshi and American volunteers. In these studies, the vaccine has proved to be completely safe with no adverse reactions, and after either two or three doses, it stimulates a gut mucosal IgA antitoxic and antibacterial immune response (including memory) comparable to that induced by cholera disease itself^{7,8}. In American volunteers who received three oral immunizations with either the B-WC vaccine or the WC vaccine component alone, vaccination protected against challenge with a dose of live cholera vibrios that caused disease in 100 percent of concurrently tested unvaccinated controls⁹.

Since 1985 the B-WC vaccine and the WC component without any B subunit (both vaccines being produced by Institut Mérieux, France and the National Bacteriological Laboratory of Sweden) have been evaluated in a large, randomized, placebo-controlled field trial involving 89,596 vaccinated adults and children in Bangladesh. Both vaccines have been found to protect against cholera for at least three years. During the initial 4-6 month period after immunization the protective efficacy of BS-WC vaccine was 85%, and was similar for all age groups¹⁰. Thereafter a protection level of ca 70% has persisted for at least three years in those over the age 5, whereas in younger children protection decreased substantially after the initial 6 months¹¹. Two doses of either vaccine were equivalent to three doses with regard to long-term protective efficacy. The B-WC vaccine had the advantages over WC vaccine alone of a greater initial protective efficacy against cholera¹⁰. In addition, the B-WC vaccine, in contrast to WC, gave substantial short-term protection against diarrhoea caused by LT as well as LT/ST enterotoxigenic *E. coli* (67% protection against all LT *E. coli* diarrhoea and 86% protection against severe disease)¹². Furthermore, the B-WC vaccine and to a lesser extent the WC vaccine, significantly decreased the over-all incidence of diarrheal disease. This impact was especially pronounced on severe, life-threatening disease which was reduced by nearly 50% in this cholera endemic area¹³.

These results with the oral B-WC and WC

vaccines differ favorably from those achieved previously using parenteral cholera vaccines. The short-term efficacy is notably higher for the oral vaccines with high-grade protection afforded also to the young children. In addition, and of greater public health significance, the duration of protection is much superior lasting at least 3 years for the oral vaccines as compared with 3–6 months for most parenteral vaccines tested.

Work to further facilitate the use of these oral vaccines is also in progress, e.g. to prepare a tablet formulation containing vaccine in combination with alkaline buffer. This would facilitate storage and distribution and probably also further increase the stability of these already rather stable vaccines for use in cholera endemic areas. By utilizing recombinant DNA technology, it also seems possible to produce the B and WC components in a single step¹⁴. This could greatly simplify large-scale production of the B-WC vaccine, reduce costs as well as allow local production of this vaccine in developing countries.

The strong, though relatively short-lasting cross-protection against diarrhea caused by LT-producing *E. coli* achieved with the B-WC vaccine also makes this vaccine a relevant immunoprophylactic agent for travellers to areas where cholera and/or *E. coli* diarrhea are prevalent. Indeed, based on these results, cholera B subunit is one component together with CFA-containing *E. coli* bacteria in a specific vaccine against *E. coli* diarrhea which is now being developed in our laboratory.

Live Attenuated Cholera Vaccines

Attenuated live organisms are appealing as oral cholera vaccine candidates because of their ability to colonize the intestine and to stimulate an immune response in a manner analogous to natural cholera infection. Through sustained delivery of antigens to the mucosal immune system a live vaccine may have a greater probability than a non-living vaccine to evoke a satisfactory immune response after only a single dose.

Recombinant DNA techniques have allowed the preparation of a series of *V. cholerae* 01 mutant strains in which the genes encoding both the A and B subunits of cholera toxin or just the A subunit have been deleted. Studies in human volunteers, have revealed that these strains evoke a protective immune response against challenge. However, even though the mutant strains were markedly attenuated compared with the parent strains they gave rise to diarrhea (usually mild) in more than one-half of the vaccinated volunteers which precluded their further

testing as potential vaccines¹⁵. Another more recently developed such strain, CVD103-HgR, which was prepared by deleting the A subunit gene from *V. cholerae* 01 strain 569B by recombinant DNA techniques, has only rarely caused any diarrheal side-effects in volunteers and yet has elicited good antibacterial and antitoxic immune responses along with significant protection against challenge. This makes CVD103-HgR an interesting candidate for expanded clinical trials now initiated in cholera endemic areas¹⁶.

B Subunits as Antigen Carriers

Recently there has been much interest in the possibility to also use cholera B subunit as carrier for foreign antigens or epitopes for the purpose of achieving a mucosal immune response. The reason for this is that, even with oral immunization the stimulation of gut-associated lymphoid tissue by many antigens is often relatively inefficient, requiring large quantities of the immunogens and yielding only modest antibody responses. Notable exceptions are cholera toxin and (in humans) its B subunit component, which are potent enteric immunogens eliciting strong mucosal immune responses. As mentioned above, this is to a large extent due to the ability of these proteins to bind avidly to GM1 ganglioside receptors on cell surfaces, and also, at least for cholera toxin, to the immunomodulating activity of these proteins. It might even be possible with this approach to achieve protective immunity also in mucosal or glandular tissues distant from the intestinal mucosa itself.

Indeed, in a recent study¹⁷, it was shown that oral administration of microgram amounts of a putative caries-protective antigen of *Streptococcus mutans* called protein antigen I/II when covalently coupled to the B subunit of cholera toxin elicited a vigorous mucosal as well as extramucosal IgA and IgG anti-streptococcal antibody response in mice. These responses, which were not achieved when antigen I/II was given in free form or coupled to bovine serum albumin, were manifested by the presence of large numbers of antibody-secreting cells in salivary glands, intestine and spleen as well as by the development of high levels of circulating antibodies.

Hybrid proteins between cholera B subunit (CTB) and foreign antigens for immuno-protection can be prepared by chemical coupling or by genetic fusion. In the genetically engineered CTB overexpression system mentioned above the possibility of obtaining CTB-derived hybrid proteins by gene fusion was described as an extra bonus¹⁴. This could allow link-

age of various peptide antigens to CTB for immunoprotection studies against various viral, bacterial and parasitic diseases. The accessibility of this approach has recently been demonstrated by the fusion and expression of a synthetic DNA sequence encoding a nontoxic decapeptide antigen, derived from the heat-stable enterotoxin, to the CTB gene¹⁸.

1. De, S. N., Enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae*. *Nature (Lond.)*, 1959, **183**, 1533-1534.
2. Panse, M. V. and Dutta, N. K., Excretion of toxin with stools of cholera patients. *J. Infect. Dis.*, 1961, **109**, 81-88.
3. Craig, J. P., A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. *Nature (Lond.)*, 1965, **207**, 614-616.
4. Ouchterlony, Ö. and Holmgren, J., (eds.) "Cholera and related Diarrheas", 43rd Nobel Symposium, Stockholm 1978, Karger, Basel, 1980.
5. Holmgren, J., Actions of cholera toxin and the prevention and treatment of cholera. *Nature (Lond.)*, 1981, **292**, 413-417.
6. Holmgren, J. and Svennerholm, A. -M., Cholera and the immune response. *Prog. Allergy*, Hanson L. A. et al (eds.), Host parasite relationships in gram-negative infections, 1983.
7. Svennerholm, A. -M., Jertborn, M., Gothefors, L., Karim A. M. M., Sack, D. A. and Holmgren, J. Mucosal antitoxic and antibacterial immunity after cholera disease and after immunization with a combined B subunit-whole cell vaccine. *J. Infect. Dis.* 1984, **149**, 884-893.
8. Jertborn, M., Immune responses in humans after oral cholera vaccination. *Ph.D. Thesis*, University of Göteborg, Sweden, 1987.
9. Black, R. E., Levine, M. M., Clements, M. L., Young, C. R., Svennerholm, A.-M. and Holmgren, J., Protective efficacy in man or killed whole vibrio oral cholera vaccine with and without the B subunit of cholera toxin. *Infect. Immun.* 1987, **55**, 1116-1120.
10. Clemens, J. D., Sack, D. A., Harris, J. R., Chakraborty, J., Khan, M. R., Stanton, B. F., Kay, B. A., Khan, M. U., Yunus, M. D., Atkinson, W., Svennerholm, A. -M. and Holmgren, J., Field trial of oral cholera vaccines in Bangladesh. *Lancet*, 1986, **i**, 124-127.
11. Clemens, J., Sack, D. A., Harris, J. R., van Loon, F., Chakraborty, J., Ahmed, F., Rao, M. R., Yunus, Md., Huda, N., Stanton, B. F., Kay, B. A., Walter, S., Eeckels, R., Svennerholm, A. -M. and Holmgren, J., Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. *Lancet*, 1990, **335**: 270-273.
12. Clemens, J. D., Sack, D. A., Harris, J. R., Chakraborty, J., Neogy, P. K., Stanton, B., Huda, N., Khan, M. U., Ansaruz-zaman, M., Yunus, M., Rao, M. R., Svennerholm, A. -M. and Holmgren, J., Cross-protection by B subunit whole-cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: Results of a large-scale field trial. *J. Infect. Dis.* 1988, **158**, 372-377.
13. Clemens, J. D., Harris, J. R., Khan, M. R., Ali, M., Yunus, M., Khan, M. U., Sack, D. A., Chakraborty, J., Stanton, B. F., Ahmed, F., Kay, B. A., Rao, M. R., Svennerholm, A. -M. and Holmgren, J., Impact of B subunit killed whole-cell and killed whole-cell-only oral vaccines against cholera upon treated diarrhoeal illness and mortality in an area endemic for cholera. *Lancet*, 1988, **i**, 1375-1379.
14. Sanchez, J. and Holmgren, J. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 481-485.
15. Levine, M. M., Kaper, J. B., Herrington, D., Losonsky, G., Morris J. G., Clements, M. L., Black, R. E., Tall, B. and Hall, R., Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. *Infect. Immun.* 1988, **56**, 161-167.
16. Levine, M. M., Herrington, D., Losonsky, G., Tall, B., Kaper, J. B., Ketley, J., Tacket, C. and Cryz, S. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. *Lancet*, 1988, **ii** 467-470.
17. Czerkinsky, C., Russell, M. W., Lycke, N., Lindblad, M. and Holmgren, J., Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. *Infect. Immun.* 1989, **57**, 1072-1077.
18. Sanchez, J. S., Svennerholm, A. -M. and Holmgren, J., Genetic fusion of a non-toxic heat-stable enterotoxin related decapeptide antigen to cholera toxin B subunit. *FEBS Lett.* 1988, **241**, 110-116.

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Reactions

De's work was not accepted at first, nor its significance realized. For example, Jenkin and Rowley, who had already invested heavily in endotoxins, were unable to repeat De's observations on the effect of cholera culture filtrate and claimed, astonishingly, that his results were due to the combined action of the old favourite, endotoxin, plus the new favourite, mucinase, plus a new-comer to the field, lactic acid. Instead of such a complicated idea, they might have been better advised to consider the far simpler idea that their cholera culture filtrates contained no exotoxin. We have seen that it is all too easy to grow a culture of the cholera vibrio producing no exotoxins. In any case, the lactic acid hypothesis was soon demolished by S. B. Formal and his colleagues at the Walter Reed Army Institute of Research in Washington, D.C.

In time, the reservations about De's ligated intestinal loop diminished as it came to find more and more use in the study of other diarrhoeal diseases as well as cholera. False positives became rarer as experience was gained in handling the small intestine, and it was found to be feasible and very convenient, to tie off as many as 24 loops in a single rabbit.

Cholera toxin, or cholera exo-enterotoxin, to be specific both about its nature and about its site of action, had at last found its way out of a maze of confusion, soon to fall into the welcoming arms of a scientific community that was ready and able to make the fullest use of it.

Excerpted from *Cholera: The American Scientific Experience, 1947-1988*
by W. E. van Heyningen and J. R. Seal

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File: USPT

May 4, 1993

US-PAT-NO: 5208041

DOCUMENT-IDENTIFIER: US 5208041 A

TITLE: Essentially pure human parathyroid hormone

DATE-ISSUED: May 4, 1993

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sindrey; Dennis R.	Etobicoke	N/A	N/A	CAX

US-CL-CURRENT: 424/562; 435/71.2, 514/2, 514/21, 530/324

CLAIMS:

We claim:

1. Essentially pure human parathyroid hormone, characterized by single peak migration when analyzed by capillary electrophoresis at 214 nm, and by an EC50 as determined in the UMR 106-based adenylate cyclase assay of not more than 2 nM.
2. Essentially pure human parathyroid hormone as defined in claim 1, in lyophilized form.
3. A method for preparing a pharmaceutical composition, which comprises the step of combining essentially pure human parathyroid hormone as defined in claim 1, with a pharmaceutically acceptable carrier.
4. A pharmaceutical composition comprising a therapeutically effective amount of essentially pure human parathyroid hormone as defined in claim 1, and a pharmaceutically acceptable carrier.
5. A pharmaceutical composition according to claim 4, wherein said carrier is an aqueous vehicle.
6. A pharmaceutical composition according to claim 5, further comprising a stability-enhancing amount of a reducing agent.
7. A pharmaceutical composition according to claim 4, further comprising a second therapeutic agent.
8. A method for purifying human parathyroid hormone, which comprises the step of fractionating a partially purified human PTH preparation by reversed phase high performance liquid chromatography with a cationic ion-pairing agent.
9. A method according to claim 8, wherein the cationic ion-pairing agent is an amine-based ion pairing agent.
10. The method according to claim 9, wherein the amine-based ion-pairing agent is triethylamine, or a salt thereof.
11. The method according to claim 10, wherein the amine-based ion-pairing agent is triethylamine phosphate.
12. The method according to claim 10 wherein the partially purified parathyroid preparation is obtained from a microbial source of human parathyroid hormone.
13. The method according to claim 12, wherein the microbial source of human parathyroid hormone is a bacterial source of human parathyroid hormone.
14. The method according to claim 13 wherein the bacterial source of human parathyroid hormone is an E. coli source of human parathyroid hormone.
15. Essentially pure human parathyroid hormone obtained by the steps of
 - i) obtaining a partially purified preparation of E. coli-produced human parathyroid hormone,
 - ii) fractionating the partially purified preparation by reversed phase high performance liquid chromatography with triethylamine or a salt thereof; and
 - iii) collecting, following the chromatographic step, essentially pure human parathyroid hormone.
16. The method according to claim 13, further comprising the subsequent step of

- Krauter, K., K. Montgomery, S.J. Yoon, J. Leblancstraceski, B. Renault, I. Marondel, V. Herdman, L. Cupelli, A. Banks, J. Lieman et al. 1995. A 2nd-generation YAC contig map of human-chromosome-12. *Nature* **377**: 321–333.
- Lee, L.G., C.R. Connell, S.L. Woo, R.D. Cheng, B.F. McArdle, C.W. Fuller, N.D. Halloran, and R.K. Wilson. 1992. DNA sequencing with dye-labeled terminators and T7 DNA-polymerase—Effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. *Nucleic Acids Res.* **20**: 2471–2483.
- Leversha, M.A. 1997. Fluorescence in situ hybridization. In *Genome mapping* (ed P.H. Dear). IRL Press, Oxford, UK.
- Marra, M.A., T.A. Kucaba, N.L. Dietrich, E.D. Green, B. Brownstein, R.K. Wilson, K.M. McDonald, L.W. Hillier, J.D. McPherson, and R.H. Waterston. 1997. High throughput fingerprint analysis of large-insert clones. *Genome Res.* **7**: 1072–1084.
- Marshall, E. 1995. A strategy for sequencing the genome 5 years early. *Science* **267**: 783–784.
- . 1998. NIH to produce a “working draft” of the genome by 2001. *Science* **281**: 1774–1775.
- McMurray, A.A., J.E. Sulston, and M.A. Quail. 1998. Short-insert libraries as a method of problem solving in genome sequencing. *Genome Res.* **8**: 562–566.
- Mungall, A.J., C.A. Edwards, S.A. Ranby, S.J. Humphray, R.W. Heathcote, C.M. Clee, C.L. East, E. Holloway, A.P. Butler, C.F. Langford et al. 1996. Physical mapping of chromosome 6: A strategy for the rapid generation of sequence-ready contigs. *DNA Seq.* **7**: 47–49.
- Murray, J.C., K.H. Buetow, J.L. Weber, S. Ludwigsen, T. Scherpbierheddema, F. Manion, J. Quillen, V.C. Sheffield, S. Sunden, G.M. Duyk et al. 1994. A comprehensive human linkage with centimorgan density. *Science* **265**: 2049–2054.
- Nagaraja, R., J. Kere, S. Macmillan, M.W.J. Masisi, D. Johnson, B.J. Molini, G.R. Halley, K. Wein, M. Trusgnich, B. Eble et al. 1994. Characterization of 4 human YAC libraries for clone size, chimerism and X-chromosome sequence representation. *Nucleic Acids Res.* **22**: 3406–3411.
- Nagaraja, R., S. MacMillan, J. Kere, C. Jones, S. Griffin, M. Schmatz, J. Terrell, M. Shomaker, C. Jermak, C. Hott et al. 1997. X chromosome map at 75-kb STS resolution, revealing extremes of recombination and GC content. *Genome Res.* **7**: 210–222.
- Olson, M.V., J.E. Dutchik, M.Y. Graham, G.M. Brodeur, C. Helms, M. Frank, M. MacCollin, R. Scheinman, and T. Frank. 1986. Random-clone strategy for genomic restriction mapping in yeast. *Proc. Natl. Acad. Sci.* **83**: 7826–7830.
- Olson, M., L. Hood, C. Cantor, and D. Botstein. 1989. A common language for physical mapping of the human genome. *Science* **245**: 1434–1435.
- Prober, J.M., G.L. Trainor, R.J. Dam, F.W. Hobbs, C.W. Robertson, R.J. Zagursky, A.J. Cocuzza, M.A. Jensen, and K. Baumeister. 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* **238**: 336–341.
- Rosenblum, B.B., L.G. Lee, S.L. Spurgeon, S.H. Khan, S.M. Menchen, C.R. Heiner, and S.M. Chen. 1997. New dye-labeled terminators for improved DNA sequencing patterns. *Nucleic Acids Res.* **25**: 4500–4504.
- Ross, M.T. and C.F. Langford. 1997. The use of flow-sorted chromosomes in genome mapping. In *Genome mapping* (ed P.H. Dear), pp. 165–184. IRL Press, Oxford, UK.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Sauer, C.G., A. Gehrig, R. Warneke-Wittstock, A. Marquardt, C.C. Ewing, A. Gibson, B. Lorenz, B. Jurklies, and B.H.F. Weber. 1997. Positional cloning of the gene associated with X-linked juvenile retinoschisis. *Nat. Genet.* **17**: 164–170.
- Sayos, J., C. Wu, M. Morra, N. Wang, X. Zhang, D. Allen, S. van Schaik, P.L.D. Notarangelo, R. Geha, M.G. Roncarolo et al. 1998. SAR, the protein encoded by the X-linked Lymphoproliferative disease gene, regulates signal transduction events induced through the co-receptor module SLAM. *Nature* **395**: 462–469.
- Shizuya, H., B. Birren, U.J. Kim, V. Mancino, T. Slepak, Y. Tachiiri, and M. Simon. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci.* **89**: 8794–8797.
- Smith, L.M., S. Fung, M.W. Hunkapiller, T.J. Hunkapiller, and L.E. Hood. 1985. The synthesis of oligonucleotides containing an aliphatic amino group at the 5' terminus: Synthesis of fluorescent DNA primers for use in DNA sequence analysis. *Nucleic Acids Res.* **13**: 2399–2412.
- Smith, L.M., R.J. Kaiser, J.Z. Sanders, and L.E. Hood. 1987. The synthesis and use of fluorescent oligonucleotides in DNA sequence analysis. *Methods Enzymol.* **155**: 260–301.
- Tabor, S. and C.C. Richardson. 1995. A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides. *Proc. Natl. Acad. Sci.* **92**: 6339–6343.
- Venter, J.C., H.O. Smith, and L. Hood. 1996. A new strategy for genome sequencing. *Nature* **381**: 364–366.

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Differences in binding affinities of human PTH(1-84) do not alter biological potency: a comparison between chemically synthesized hormone, natural and mutant forms.**Olstad OK, Morrison NE, Jemtland R, Juppner H, Segre GV, Gautvik KM**

Institute of Medical Biochemistry, University of Oslo, Norway.

Related Resources

The purpose of this study was to evaluate receptor binding affinities and biological properties in vitro and in vivo of various recombinant hPTH(1-84) forms representing the natural hormone and a mutagenized hPTH form, [Gln26]hPTH(1-84) (QPTH), after expression in *E. coli* and *Saccharomyces cerevisiae*. In LLC-PK1 cells stably transformed with the rat PTH/PTHrP receptor, chemically synthesized hPTH(1-84) and QPTH showed a reduced binding affinity (apparent K_d 18 and 23 nM, respectively) than the recombinant, hPTH(1-84) (apparent K_d 9.5 nM). All recombinant hPTH forms showed a similar potency to stimulate cellular cAMP production (EC₅₀ 1.5 nM) and significantly better than chemically synthesized hPTH (EC₅₀ 5.7 nM). All hormone forms showed an about equipotent activity in causing elevation in serum calcium, increased excretion of urine phosphate, and cAMP. Thus, the natural recombinant PTH forms showed higher binding affinities and adenylate cyclase activation potencies in LLC-PK1 cells, but the reduced receptor binding affinity exerted by QPTH did not transcend differences in cAMP generation and in vivo biological activities.

PMID: 7854979, UI: 95158218

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overlapping YACs ordered on the basis of shared landmark content [chiefly sequence-tagged sites (STSs) (Olson et al. 1989)]. The contigs were positioned using markers that were also present in the available genetic (Murray et al. 1994; Dib et al. 1996) or radiation hybrid (RH) maps of the human genome. The average STS spacing of these combined maps is ~1/250 kb for the whole genome maps (Chumakov et al. 1995; Hudson et al. 1995), and up to 1/70 kb for selected single chromosomes (Chumakov et al. 1992; Foote et al. 1992; Collins et al. 1995; Doggett et al. 1995; Gemmill et al. 1995; Krauter et al. 1995; Bouffard et al. 1997; Nagaraja et al. 1997). Although long-range continuity was obtained with these maps, human YACs were not considered suitable substrates for large-scale genomic sequencing in general, because of the high degree of chimerism and the instability observed in a large proportion of the YAC clones (Green et al. 1991; Nagaraja et al. 1994). Therefore, these early maps must be converted to a practicable reagent to support the large-scale sequencing of the human genome. More recently, RH mapping has been used to construct a physical map of the human genome, containing 30,181 unique gene-based markers (Deloukas et al. 1998). Mapped markers from both the gene map and the YAC based maps form the basis for the construction of bacterial clone maps for sequencing.

For the human genome, an important breakthrough for building sequence-ready maps was the development of the new large insert (up to at least 200 kb) bacterial- and P1-artificial chromosome (BAC and PAC, respectively) (Shizuya et al. 1992; Ioannou et al. 1994) cloning systems and the con-

struction of comprehensive human genomic libraries. There are currently a number of libraries available, with a combined 60-fold representation (see Table 2), and more are presently under construction (P. de Jong and U.-J. Kim, pers. comm.). Clones from these libraries are stable and contain few rearrangements on the basis of the data available so far (Shizuya et al. 1992; Ioannou et al. 1994).

Our strategy for constructing sequence-ready maps (Fig. 1) consists of screening for bacterial clones using a high density of STSs (15/Mb on average). These markers were initially taken from the available YAC maps (for chromosomes 2, 7, 22, and X) and were supplemented with all other markers in the public domain. For chromosomes 1, 6, and 20, markers derived from ESTs (Hillier et al. 1996) and flow-sorted chromosomes (Ross and Langford 1997) were used together with existing markers to assemble integrated maps by RH mapping (Cox et al. 1990; Walter et al. 1994; Mungall et al. 1996) (maps are available at <http://www.sanger.ac.uk/HGP/Rhmap>). The bacterial clones are assembled into contigs by comparative restriction fingerprint analysis (Coulson et al. 1986; Olson et al. 1986; Gregory et al. 1997; Marra et al. 1997) and landmark content mapping (Green and Olson 1990). The nonuniform distribution of markers means that many gaps remain at this stage, but contigs are then extended and joined by the generation of new markers at their ends. Some larger gaps are also filled using region-specific probes generated from bridging YAC clones. A minimally overlapping subset of the bacterial clones is selected for sequencing by manual inspection of the overlapping fingerprints relating to each clone. All selected clones are verified by ensuring that all available restriction patterns, landmark content, and fluorescent in situ hybridization data for each clone are entirely consistent. This general approach has two advantages over other methods such as whole-genome shotgun (Weber and Myers 1997; Venter et al. 1998) or end-sequencing BAC clones followed by walking (Venter et al. 1996). Neither of the latter approaches can be coordinated efficiently to minimize overlap between collaborating groups, nor do they make use of all the available map information that

Table 2. Bacterial Clone Libraries That Are Currently Available for Human Sequencing

Library	Type	Source DNA	Number of clones	Average size (kb)	Coverage (x)
RPCI-1, 3, 4, 5	PAC	male	438,737	114	16
RPCI-6	PAC	female	87,897	135	4
RPCI-11	BAC	male	437,034	174	25.3
BAC library D	BAC	male	280,000	170	16
Library	Vector	Site	URL		
RPCI-1, 3, 4, 5	pCYPAC2	<i>Mbol/Bam</i> HI	http://bacpac.med.buffalo.edu		
RPCI-6	pPAC4	<i>Mbol/Bam</i> HI	http://bacpac.med.buffalo.edu		
RPCI-11	pBACe3.6	<i>Eco</i> RI	http://bacpac.med.buffalo.edu		
BAC library D	pBeloBAC11	<i>Hind</i> III or <i>Eco</i> RI	http://www.tree.caltech.edu		

RPCI libraries are from the Roswell Park Cancer Institute Group of Pieter de Jong. BAC library D is from the Caltech group of Ung-Jin Kim, Hiroaki Shizuya, and Melvin Simon.

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Recombinant human [Cys281]insulin-like growth factor-binding protein 2 inhibits both basal and insulin-like growth factor I-stimulated proliferation and collagen synthesis in fetal rat calvariae.

Feyen JH, Evans DB, Binkert C, Heinrich GF, Geisse S, Kocher HP

Department of Endocrinology, Sandoz Pharma Limited, Basel, Switzerland.

Related Resources

It is recognized that insulin-like growth factors (IGFs) are bound to specific high-affinity insulin-like growth factor-binding proteins (IGFBPs). The role of IGFBPs in bone metabolism is not well established. The effect of recombinant human [Cys281]IGFBP-2 ([Cys281]rhIGFBP-2) on bone formation in 21-day-old fetal rat calvariae was investigated. [Cys281]rhIGFBP-2 was expressed in and purified from conditioned medium of a clonal Chinese hamster ovary cell line. IGF-I-stimulated cell proliferation was inhibited dose dependently by [Cys281]rhIGFBP-2, with half-maximal inhibition observed at 2×10^{-8} M. Suppression of the IGF-I-stimulated DNA synthesis was observed at an apparent dose ratio of 1:10. [Cys281]rhIGFBP-2 (10^{-6} M) also inhibited the basal incorporation of [3H]thymidine into DNA by up to 45%. Insulin-stimulated cell proliferation was not affected in the presence of the binding protein. In addition, [Cys281]rhIGFBP-2 inhibited bone collagen synthesis under basal and IGF-I-stimulated conditions. In contrast, [Cys281]rhIGFBP-2 did not alter the parathyroid hormone-stimulated bone cell proliferation rate. In conclusion, binding of hIGF-I to rhIGFBP-2 results in an inhibition of the actions of free IGF-I on bone cell replication and matrix synthesis. Parathyroid hormone-stimulated cell proliferation is not mediated by an increase in free IGFs.

PMID: 1717466, UI: 92011744

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progress so far, we are confident that the prospects are excellent for sequencing the rest of the human genome by 2005, as part of an international collaborative effort involving many laboratories (see Table 1). In this article, we describe our work to date and discuss the problems we have solved so far and the issues that still must be addressed.

Determining the Human Genome Sequence

A key to the success of the *C. elegans* sequencing

project was the availability of an almost complete physical map of the genome in overlapping cosmids that were joined by bridging yeast artificial chromosome (YAC) clones (Coulson et al. 1986, 1988). A minimally overlapping subset of cosmids was selected from the available contigs for most of the sequencing, and this is now being complemented by shotgun sequencing of selected YACs to finish the project.

In contrast, the most comprehensive physical maps of the human genome have comprised sets of

Table 1. International Consortium of Laboratories Involved in Large-Scale Sequencing

Laboratory	URL
Applied Biosystems Division (ABD) of Perkin-Elmer Corp., CA USA	http://www.abc.wustl.edu/cgm/
Baylor College of Medicine (BCM), Houston, TX USA	http://gc.bcm.tmc.edu:8088/cgi-bin/seq/home/
The California Institute of Technology, Pasadena, CA USA	http://www.tree.caltech.edu/
The Cold Spring Harbor Laboratory, NY USA	http://clio.cshl.org/
The DNA Database of Japan (DDBJ)	http://www.ddbj.nig.ac.jp/
The European Bioinformatics Institute (EBI)	http://www.ebi.ac.uk/
Genome Sequencing Centre (GSC), Washington University School of Medicine, St. Louis, MO USA	http://genome.wustl.edu/gsc/
Genoscope, Evry, France	http://www.genoscope.cns.fr/
The Institute for Genomic Research (TIGR)	http://www.tigr.org/tdb/humgen/progressmap.html
The Institute of Molecular Biotechnology (IMB), Jena, Germany	http://genome.imb-jena.de/
Japan Science and Technology Corporation (JST), Tokyo, Japan	http://www-alis.tokyo.jst.go.jp/HGShome.html
The Joint Genome Institute (JGI), U.S. Dept. of Energy, Berkeley, Livermore, CA USA	http://www.jgi.doe.gov/
The Max-Planck-Institut für Molekulare Genetik, Berlin, Germany	http://www/mpimg-berlin-dahlem.mpg.de/
The Medical Research Council (MRC), London, UK	http://www.mrc.ac.uk
The National Center for Biotechnology Information (NCBI), Bethesda, MD USA	http://www.ncbi.nlm.nih.gov/
The National Human Genome Research Institute (NHGRI), Bethesda, MD USA	http://www.nhgri.nih.gov/
National Institute of Genetics, Shizuoka, Japan	http://www.nig.ac.jp/
Roswell Park Cancer Institute (RPCI), Buffalo, NY USA	http://rpci.med.buffalo.edu/
The Sanger Centre (SC), Hinxton, UK	http://www.sanger.ac.uk/
Stanford Human Genome Center (SHGC), Stanford, CA USA	http://www.-shgc.stanford.edu/
The University of Oklahoma (UO), OK USA	http://www.genome.ou.edu/maps/ch22.html
University of Texas SouthWestern (UTSW) Medical Center, Dallas, TX USA	http://gestec.swmed.edu/
The University of Washington (UW), Seattle, WA USA	http://www.genome.washington.edu
The Wellcome Trust, London, UK	http://www.wellcome.ac.uk/
The Whitehead Institute (WI)	http://www-genome.wi.mit.edu/

The international consortium was formed in 1996, with the aim of determining the complete sequence of the human genome. The consortium unanimously endorsed the Bermuda statement that "all human genomic sequence information should be freely available and in the public domain in order to encourage research and development and to maximize its benefit to society." The consortium is still evolving (laboratories adopting the data release policy can join at any time), and as a result this list is not exhaustive.

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Recombinant human parathyroid hormone synthesized in Escherichia coli. Purification and characterization.**Rabbani SA, Yasuda T, Bennett HP, Sung WL, Zahab DM, Tam CS, Goltzman D, Hendy GN**

Department of Medicine, McGill University, Montreal, Quebec, Canada.

Related Resources

Recombinant human parathyroid hormone (hPTH) was expressed in Escherichia coli harboring a plasmid containing a synthetic human parathyroid hormone gene under the control of the E. coli lac promoter. Three major forms of the hormone were isolated by acid extraction and purified to homogeneity by high performance liquid chromatography. By amino acid analysis and NH₂-terminal sequencing, these were identified as hPTH-(1-84), formyl-methionyl-hPTH-(1-84), and hPTH-(8-84). The recombinant hPTH-(1-84) was immunologically indistinguishable from a World Health Organization standard of extracted native hPTH-(1-84). Recombinant hPTH-(1-84) was also bioactive in renal and skeletal adenylate cyclase assays. In the skeletal bioassay performed in UMR 108 osteosarcoma cells its activity was identical to that of an hPTH-(1-84) standard. In this bioassay, formyl-methionyl-hPTH-(1-84) had 10% of the activity of hPTH-(1-84) and hPTH-(8-84) was inactive. The results demonstrate the importance of isolating hPTH-(1-84) from other recombinant forms and metabolites to achieve full hormonal bioactivity and indicate that purified recombinant hPTH-(1-84) can thereby be obtained which should be a useful source of hormone for both basic and clinical studies.

PMID: 3275665, UI: 88087263

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Venter, J.C., M.D. Adams, G.G. Sutton, A.R. Kerlavage, H.O. Smith, and M. Hunkapiller. 1998. Shotgun sequencing of the human genome. *Science* **280**: 1540-1542.

Wadman, M. 1998. Human genome deadline cut by two years. *Nature* **395**: 207.

Walter, M.A., D.J. Spillett, P. Thomas, J. Weissenbach, and P.N. Goodfellow. 1994. A method for constructing radiation hybrid maps of whole genomes. *Nat. Genet.* **7**: 22-28.

Waterston, R. and J. Sulston. 1998. Human genome project: Reaching the finish line. *Science* **282**: 53-54.

Weber, J.L. and E.W. Myers. 1997. Human whole-genome shotgun sequencing. *Genome Res.* **7**: 401-409.

Wooster, R., G. Bignell, J. Lancaster, S. Swift, S. Seal, J. Mangion, N. Collins, S. Gregory, C. Gumbs, G. Micklem et al. 1995. Identification of the breast-cancer susceptibility gene BRCA2. *Nature* **378**: 789-792.

Received July 7, 1998; accepted in revised form October 6, 1998.

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PubMed Services

Expression and characterization of a recombinant human parathyroid hormone secreted by Escherichia coli employing the staphylococcal protein A promoter and signal sequence.**Hogset A, Blingsmo OR, Saether O, Gautvik VT, Holmgren E, Hartmanis M, Josephson S, Gabrielsen OS, Gordeladze JO, Alestrom P, et al**

Related Resources

Institute of Medical Biochemistry, University of Oslo, Blindern, Norway.

Human parathyroid hormone (hPTH) is a peptide hormone consisting of 84 amino acids (hPTH(1-84)). Employing the promoter and signal sequence of Staphylococcus aureus-protein A we have expressed hPTH in Escherichia coli. The expressed proteins are excreted to the growth medium, allowing for rapid and easy purification of the desired products. By amino acid sequence analysis and mass spectrometry, we have shown that the major excreted product is correctly processed human identical hPTH(1-84). The purified recombinant hPTH(1-84) stimulates adenylate cyclase activity in rat osteosarcoma cell membranes to exactly the same extent as synthetic parathyroid hormone standards, indicating that the recombinant product has full biological activity.

PMID: 2185244, UI: 90237029

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1992. Continuum of overlapping clones spanning the entire human chromosome 21q. *Nature* **359**: 380-387.
- Chumakov, I.M., P. Rigault, I. Le Gall, C. Bellanné-Chantelot, A. Billault, S. Guillou, P. Soularue, G. Guasconi, E. Poullier, I. Gros et al. 1995. A YAC contig map of the human genome. *Nature* **377**: 175-298.
- Coffey, A.J., R.A. Brooksbank, O. Brandau, T. Oohashi, G.R. Howell, J.M. Bye, A.P. Cahn, J. Durham, P. Heath et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat. Genet.* **20**: 129-135.
- Collins, J.E., C.G. Cole, L.J. Smith, C.L. Garrett, M.A. Leversha, C.A. Soderlund, G.L. Maslen, L.A. Everett, K.M. Rice, A.J. Coffey et al. 1995. A high-density YAC contig map of human-chromosome-22. *Nature* **377**: 367-379.
- Coulson, A., J. Sulston, S. Brenner, and J. Karn. 1986. Toward a physical map of the genome of the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci.* **83**: 7821-7825.
- Coulson, A., R. Waterston, J. Kiff, J. Sulston, and Y. Kohara. 1988. Genome linking with yeast artificial chromosomes. *Nature* **335**: 184-186.
- Cox, D.R., M. Burmeister, E.R. Price, S. Kim, and R.M. Myers. 1990. Radiation hybrid mapping: A somatic cell genetic method for constructing high-resolution maps of mammalian chromosomes. *Science* **250**: 245-250.
- Dear, S., R. Durbin, L. Hillier, G. Marth, J. Thierry-Mieg, and R. Mott. 1998. Sequence assembly with CAFTOOLS. *Genome Res.* **8**: 260-267.
- Deloukas, P., G.D. Schuler, G. Gyapay, E.M. Beasley et al. 1998. A physical map of 30,000 human genes. *Science* **282**: 744-746.
- Dib, C., S. Faure, C. Fizames, D. Samson, N. Drouot, A. Vignal, P. Millasseau, S. Marc, J. Hazan, E. Seboun et al. 1996. A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* **380**: 152-154.
- Doggett, N.A., L.A. Goodwin, J.G. Tesmer, L.J. Meincke, D.C. Bruce, L.M. Clark, M.R. Altherr, A.A. Ford, H.C. Chi, B.L. Marrone et al. 1995. An integrated physical map of human-chromosome-16. *Nature* **377**: 335-365.
- Dunham, I., K. Dewar, U.-J. Kim, and M.T. Ross. 1998. Bacterial cloning systems. In *Genome analysis* (ed. B. Birren, E. Green, P. Hieter, S. Klapholz, and R. Myers). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (In press.)
- Durbin, R. and J. Thierry-Mieg. 1991. A C. elegans database. WWW: <http://www.sanger.ac.uk/Software/Acedb/>.
- Everett, L.A., B. Glaser, J.C. Beck, J.R. Idol, A. Buchs, M. Heyman, F. Adawi, E. Hazani, E. Nassir, A.D. Baxevanis, V.C. Sheffield, and E.D. Green. 1997. Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat. Genet.* **17**: 411-422.
- Ewing, B. and P. Green. 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* **8**: 186-194.
- Ewing, B., L. Hillier, M.C. Wendl, and P. Green. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **8**: 175-185.
- Foote, S., D. Vollrath, A. Hilton, and D.C. Page. 1992. The human Y chromosome: Overlapping DNA clones spanning the euchromatic region. *Science* **258**: 60-66.
- Gemmell, R.M., I. Chumakov, P. Scott, B. Waggoner, P. Rigault, J. Cypser, Q. Chen, J. Weissenbach, K. Gardiner, H. Wang et al. 1995. A 2nd-generation YAC contig map of human-chromosome-3. *Nature* **377**: 299-319.
- Gordon, D., C. Abajian, and P. Green. 1998. Consed: A graphical tool for sequence finishing. *Genome Res.* **8**: 195-202.
- Green, E.D. and M.V. Olson. 1990. Chromosomal region of the cystic fibrosis gene in yeast artificial chromosomes: A model for human genome mapping. *Science* **250**: 94-98.
- Green, E.D., H.C. Riethman, J.E. Dutchik, and M.V. Olson. 1991. Detection and characterization of chimeric yeast artificial-chromosome clones. *Genomics* **11**: 658-669.
- Green, P. 1997. Against a whole-genome shotgun. *Genome Res.* **7**: 410-417.
- Gregory, S.G., G.R. Howell, and D.R. Bentley. 1997. Genome mapping by fluorescent fingerprinting. *Genome Res.* **7**: 1162-1168.
- Hillier, L., G. Lennon, M. Becker, M.F. Bonaldo, B. Chiapelli, S. Chisoe, N. Dietrich, T. Dubuque, A. Favello, W. Gish et al. 1996. Generation and analysis of 280,000 human expressed sequence tags. *Genome Res.* **6**: 807-828.
- Huang, X.C., M.A. Quesada, and R.A. Mathies. 1992. DNA sequencing using capillary array electrophoresis. *Anal. Chem.* **64**: 2149-2154.
- Hudson, T.J., L.D. Stein, S.S. Gerety, J. Ma, A.B. Castle, J. Silva, D.K. Slonim, R. Baptista, L. Kruglyak, S.-H. Zu, X. Hu et al. 1995. An STS-based map of the human genome. *Science* **270**: 1945-1954.
- Ioannou, P.A., C.T. Amemiya, J. Garnes, P.M. Kroisel, H. Shizuya, C. Chen, M.A. Batzer, and P.J. de Jong. 1994. A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat. Genet.* **6**: 84-89.
- Ju, J., C. Ruan, C.W. Fuller, A.N. Glazer, and R.A. Mathies. 1995. Fluorescence energy transfer dye-labeled primers for DNA sequencing and analysis. *Proc. Natl. Acad. Sci.* **92**: 4347-4351.

stably expressed recombinant receptor.

Endocrinology. 1996 Jul;137(7):2748-57.

PMID: 8770894; UI: 96366780

- ☐ 7 : Inomata N, Akiyama M, Kubota N, Juppner H. Related Articles

Characterization of a novel parathyroid hormone (PTH) receptor with specificity for the carboxyl-terminal region of PTH-(1-84).

Endocrinology. 1995 Nov;136(11):4732-40.

PMID: 7588200; UI: 96042468

- ☒ 8 : Born W, Loveridge N, Petermann JB, Kronenberg HM, Potts JT Jr, Fischer JA. Related Articles

Inhibition of parathyroid hormone bioactivity by human parathyroid hormone (PTH)-(3-84) and PTH-(8-84) synthesized in Escherichia coli.

Endocrinology. 1988 Oct;123(4):1848-53.

PMID: 3046927; UI: 88328934

- ☐ 9 : Pines M, Fukayama S, Costas K, Meurer E, Goldsmith PK, Xu X, Muallem S, Behar V, Chorev M, Rosenblatt M, Tashjian AH Jr, Suva LJ. Related Articles

Inositol 1-,4-,5-trisphosphate-dependent Ca²⁺ signaling by the recombinant human PTH/PTHrP receptor stably expressed in a human kidney cell line.

Bone. 1996 Apr;18(4):381-9.

PMID: 8726398; UI: 96291464

- ☐ 10 : Takasu H, Bringham FR. Related Articles

Type-1 parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptors activate phospholipase C in response to carboxyl-truncated analogs of PTH(1-34).

Endocrinology. 1998 Oct;139(10):4293-9.

PMID: 9751512; UI: 98422132

- ☐ 11 : Iida-Klein A, Guo J, Xie LY, Juppner H, Potts JT Jr, Kronenberg HM, Bringham FR, Abou-Samra AB, Segre GV. Related Articles

Truncation of the carboxyl-terminal region of the rat parathyroid hormone (PTH)/PTH-related peptide receptor enhances PTH stimulation of adenylyl cyclase but not phospholipase C.

J Biol Chem. 1995 Apr 14;270(15):8458-65.

PMID: 7721741; UI: 95238327

- ☒ 12 : Zanelli JM, Lane E, Kimura T, Sakakibara S.  Related Articles

Biological activities of synthetic human parathyroid hormone (PTH) 1-84 relative to natural bovine 1-84 PTH in two different in vivo bioassay systems.

Endocrinology. 1985 Nov;117(5):1962-7.

PMID: 2995003; UI: 86004480

- ☒ 13 : Goud NA, McKee RL, Sardana MK, DeHaven PA, Huelar E, Syed MM, Goud RA, Gibbons SW, Fisher JE, Levy JJ, et al. Related Articles

Solid-phase synthesis and biologic activity of human parathyroid hormone (1-84).

J Bone Miner Res. 1991 Aug;6(8):781-9.

PMID: 1664643; UI: 92151813

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/18973

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/53, 33/567; C12N 5/00, 5/02

US CL : 435/7.1, 7.2, 325; 530/300, 350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.2, 325; 530/300, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GenEmbl; N_Geneseq_36; Issued_Patents_NA; EST; A_Geneseq_36; Issued_Patents_AA; PIR; SwissProt_38; SPTREMBL_12

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHOEPP et al. Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. TIPS. December 1990. Vol. 11, pages 508-515, see entire document.	1-26
A	BRAKEMAN et al. Homer: a protein that selectively binds metabotropic glutamate receptors. Nature. March 1997. Vol. 386, pages 284-288, see entire document.	1-71
X	A_Geneseq_36. DAGGETT et al. 'New DNA encoding human metabotropic glutamate receptor subtypes - and related proteins, probes, RNA, transformed cells and antibodies, useful in treatment, diagnosis and identification of specific modulators,' July 1998.	59
X	Database: SPTREMBL_12. NAGASE et al. 'Prediction of the coding sequences of unidentified human genes. VII. The complete sequence of 100 new cDNA clones from brain which can code for large proteins in vivo,' January 1998.	63
X	Database: SPTREMBL_12. NAGASE et al. 'Prediction of the coding sequences of unidentified human genes. VII. The complete sequence of 100 new cDNA clones from brain which can code for large proteins in vivo,' January 1998.	65

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Secretion of human parathyroid hormone from rat pituitary cells infected with a recombinant retrovirus encoding preproparathyroid hormone.**Hellerman JG, Cone RC, Potts JT Jr, Rich A, Mulligan RC, Kronenberg HM**

Related Resources

In order to study the functions of precursors to secreted proteins, we expressed cloned DNA encoding human preproparathyroid hormone (preproPTH) in rat pituitary cells. We first constructed a recombinant plasmid containing human preproPTH cDNA and retroviral control signals. This recombinant plasmid was transfected into psi-2 cells, a packaging cell line that produces Moloney murine leukemia viral particles containing no retroviral RNA. The transfected psi-2 cells generated helper-free recombinant retrovirus encoding preproPTH, and this recombinant retrovirus was used to infect GH4 rat pituitary cells. Clonal lines of the infected GH4 cells contained copies of the recombinant provirus stably integrated via the long terminal repeats, and the expected RNA transcripts of proviral DNA accumulated in the cytoplasm, although no infectious virus was produced. The infected cells synthesized and processed preproPTH appropriately and secreted PTH in response to thyrotropin-releasing hormone, a secretagogue for GH4 cells. Use of recombinant retrovirus permits the introduction of DNA encoding normal and mutant secreted proteins into a number of cell types specialized for secretion. Analysis of the fate of the resultant proteins will help define the specific molecular interactions involved in transmembrane transport and processing of precursor proteins.

PMID: 6089196, UI: 84298134

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Sequence: 1 ALTPSPSPFRD 10

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SUMMARIES

Result No.	Score	Match	Length	DB ID	Description
1	55	100.0	1180	1 R64253	Human mglur5a. New
2	55	100.0	1194	1 R42199	Hsmglur1. New huma
3	55	100.0	1199	1 R25080	GLU-C-R subtype 1a
4	55	100.0	1212	1 R64254	Human mglur5b. New
5	55	100.0	1219	1 W25763	Amino acid sequenc
6	40	72.7	22	1 R28278	Sequence of cowpea
7	40	72.7	40	1 R28275	Sequence of the am
8	40	72.7	40	1 R89940	Cowpea mosaic viru
9	38	69.1	220	1 W69714	Streptomyces clavu
10	36	65.5	9	1 W87452	Peptide determined
11	36	65.5	114	1 W73409	Human secreted pro
12	36	65.5	129	1 W88506	Human liver clone
13	35	63.6	328	1 W82560	Rice glycogenin-11
14	35	63.6	965	1 W18304	Photorhabdus lumin
15	35	63.6	965	1 W56567	Toxin TccA, encode
16	34	61.8	219	1 W31759	A novel human h4-1
17	34	61.8	219	1 W92523	Human h4-1BBSV rec
18	34	61.8	219	1 W92524	Human h4-1BBSV rec
19	34	61.8	255	1 R64197	Human 4-1BB polye
20	34	61.8	255	1 R70977	H4-1BB receptor pr
21	34	61.8	255	1 R74087	Human receptor ind
22	34	61.8	255	1 W04174	Human receptor H4-
23	34	61.8	255	1 W26558	Human 4-1BB recept
24	34	61.8	281	1 W72190	HSV-2 strain SB5 C
25	34	61.8	354	1 W72045	HSV-2 strain SB5 C
26	34	61.8	354	1 W72122	HSV-2 strain SB5 C
27	34	61.8	393	1 R66244	Bifunctional uroki
28	34	61.8	396	1 R66246	Bifunctional uroki
29	34	61.8	397	1 R66248	Bifunctional uroki
30	34	61.8	415	1 W30193	Epidermal differen
31	34	61.8	540	1 W19632	Human osteo activi
32	34	61.8	540	1 W61600	Human extracellular
33	34	61.8	540	1 W94282	Human extracellular

34 34 61.8 902 1 W02250 Human transcriptio
35 34 61.8 933 1 R33212 Sequence of the pl
36 33 60.0 7 1 Y04423 Interleukin 6 bind
37 33 60.0 16 1 W25454 Grb2 N-terminal SH
38 33 60.0 19 1 W37818 Human SARP2 non-fr
39 33 60.0 52 1 R28277 Sequence of cowpea
40 33 60.0 52 1 R89942 Foot and mouth dis
41 33 60.0 314 1 W37817 Human secreted apo
42 33 60.0 385 1 R10692 Cephalosporin anti
43 33 60.0 541 1 W67820 Human secreted pro
44 33 60.0 627 1 W62552 Shiitake tyrosinas
45 33 60.0 627 1 W62553 Protein of the spe

ALIGNMENTS

RESULT 1
R64253
ID R64253 standard; Protein; 1180 AA.
AC R64253;
DT 21-JUL-1995 (first entry)
DE Human mglur5a.
KW Metabotropic glutamate receptor; mglur5; mglur5a; hippocampus;
KW cerebellum; pCMV-T7-3.
OS Homo sapiens
PN W09429449-3
PD 32-DEC-1994.
PF 03-JUN-1994; U06273.
PR 04-JUN-1993; US-072574.
PA (SALK) SALK INST BIOTECHNOLOGY IND ASSOC.
PI Daggett L, Ellis SB, Hess SD, Johnson EC, Liaw C;
PI Pontsler A;
DR WPI; 95-036478/05.
DR N-PSDB; Q80419.
PT New DNA encoding human metabotropic glutamate receptor
PT sub-type(s) - and related proteins, probes, RNA, transformed
PT cells and antibodies, useful in treatment, diagnosis and
PT identification of specific modulators
PS Claim 15; Page 90-95; 125pp; English.
CC A human hippocampus cDNA library was screened with DNA encoding
CC the rat mglur1 receptor. An isolated clone was used to screen
CC human cerebellum cDNA libraries to isolate additional clones. 3
CC 3 splice variants of the human mglur5 transcript, mglur5a (given
CC in Q80419), mglur5b (Q80520) and mglur5c (Q80421), encoding the
CC proteins given in R64253-55, respectively, were constructed by
CC ligating different overlapping clones. Coding sequences are linked
CC to regulatory elements of pCMV-T7-3 for expression in mammalian
CC cells.
SQ Sequence 1180 AA;

Query Match 100.0%; Score 55; DB 1; Length 1180;
Best Local Similarity 100.0%; Pred. No. 0.34;
Matches 10; Conservative 0; Mismatches 0; Indels 0; Gaps 0;

QY 1 ALTPSPSPFRD 10
| | | | | | | | | |
Db 1130 ALTPSPSPFRD 1139

RESULT 2
R42199
ID R42199 standard; Protein; 1194 AA.
AC R42199;
DT 16-MAY-1994 (first entry)
DE Hsmglur1.
KW Human; metabotropic; glutamate receptor; Hsmglur1; drug; stroke;
KW Alzheimer's disease; head trauma; epilepsy.
OS Homo sapiens.
PN EP-569240-A.
PD 10-NOV-1993.
PF 06-MAY-1993; 303520.

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